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(54) Title: TARGET CELL–SPECIFIC ADENOVIRAL VECTORS CONTAINING E3 AND METHODS OF USE THEREOF

(57) Abstract

The invention provides adenoviral vectors (preferably replication competent) comprising both an E3 sequence and at least one adenoviral gene under transcriptional control of a target cell–specific transcriptional response element. These vectors display significantly improved cytotoxicity, which is especially useful in the cancer context, in which selective destruction of target cells is desirable. The invention further provides host cells comprising the vectors. The invention further provides methods of using the adenoviral vectors.

TARGET CELL-SPECIFIC ADENOVIRAL VECTORS CONTAINING E3 AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority benefit of U.S. Provisional Patent Application No.60/114,262, filed December 30, 1998. The priority application is hereby incorporated herein by reference in its entirety.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

(Not applicable)

TECHNICAL FIELD

This invention relates to the field of adenoviral vectors and transfection. More specifically, the invention relates to target cell-specific adenoviral vectors containing E3.

BACKGROUND ART

In spite of numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Neoplasia resulting in benign tumors can usually be completely cured by removing the mass surgically. If a tumor becomes malignant, as manifested by invasion of surrounding tissue, it becomes much more difficult to eradicate. Once a malignant tumor metastasizes, it is much less likely to be eradicated.

A major, indeed the overwhelming, obstacle to cancer therapy is the problem of selectivity; that is, the ability to inhibit the multiplication of tumor cells, while leaving unaffected the function of normal cells. For example, in prostate cancer therapy, the therapeutic ratio, or ratio of tumor cell killing to normal cell killing of traditional tumor chemotherapy, is only 1.5:1. Thus, more effective treatment methods and pharmaceutical compositions for therapy and prophylaxis of neoplasia are needed.

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One possible treatment approach for many of these cancers is gene therapy, whereby a gene of interest is introduced into the malignant cell. Various viral vectors, including adenoviral vectors, have been developed as vehicles for gene therapy. The virtually exclusive focus in development of adenoviral vectors for gene therapy is use of

5   adenovirus merely as a vehicle for introducing the gene of interest, not as an effector in itself. Replication of adenovirus has been viewed as an undesirable result. In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently expressed in

10   transduced cells, eliciting cellular immunity to the virus-infected cells. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes, suicide genes, or genes which convert prodrug to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle as well as the transient nature of gene expression.

15   Adenovirus can cause persistent infections in humans and animals. The strategies of C type adenovirus (type Ad2 and Ad5) for evading host immune recognition are many, and generally involve E3, a delayed early transcription unit whose transcription is induced by the 289R E1A protein. During early stages of infection, the E3 promoter drives expression of nine alternatively spliced mRNAs that are polyadenylated at one of two sites,

20   E3A and E3B. Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199 (Pt.1):237-274. None of the E3 proteins is apparently required for adenovirus replication in cultured cells or in the lungs of hamsters or cotton rats, but they appear to play a role in evasion of host immune surveillance.

Six proteins which are encoded by the Ad-E3 region have been identified and

25   characterized: (1) a 19-kDa glycoprotein (gp19k) is one of the most abundant adenovirus early proteins, and is known to inhibit transport of the major histocompatibility complex class I molecules to the cell surface, thus impairing both peptide recognition and clearance of Ad-infected cells by cytotoxic T lymphocytes (CTLs); (2) E3 14.7k protein and the E3 10.4k/14.5k complex of proteins inhibit the cytotoxic and inflammatory responses mediated

30   by tumor necrosis factor (TNF); (3) E3 10.4k/14.5k protein complex downregulates the epidermal growth factor receptor, which may inhibit inflammation and activate quiescent

infected cells for efficient virus replication; (4) E3 11.6k protein (adenoviral death protein, ADP) from adenovirus 2 and 5 appears to promote cell death and release of virus from infected cells. Other studies have indicated that the E3-encoded 10.4K/14.5K complex proteins down-modulate the apoptosis receptor Fas/Apo-1. Elsing and Burger (1998) *Proc.*

5   *Natl. Acad. Sci. USA* 95:10072-10077; and Shisler et al. (1997) *J. Virol.* 71:8299-8306. The functions of three E3-encoded proteins — 3.6k, 6.7k, and 12.5k — are currently unknown. Wold et al. (1995).

Traditionally, the pervasive dogma regarding the role of E3 in adenoviral vectors for gene therapy was that E3 should be deleted. E3 was viewed as non-essential for

10   replication, and its deletion allowed insertion of foreign genes. Indeed, until quite recently, all adenoviral vectors lacked the E3 region.

More recently, it has been demonstrated that incorporation of E3 genes in the engineered adenovirus reduces the antiviral immune response and prolongs expression of foreign genes delivered by adenoviral vectors. It was shown that insertion of E3 genes in

15   recombinant adenovirus facilitates re-administration of a functional vector for long-term gene expression and correction of an inherited metabolic disorder. Horwitz et al. (1995) *Curr. Topics Microbiol. Immunol.* 199(Pt 1):195-211. Other studies have indicated that, while expression from E3-deleted vectors is essentially turned off eight weeks after gene transfer, an E3-containing vector supported transgene expression with therapeutic levels of

20   human factor IX *in vivo* for more than 4 months. Poller et al. (1996) *Gene Ther.* 3:521-530. The enhanced stability was attributed to efficient E3 region-mediated suppression of the host's antiviral immune response. More recently, it was demonstrated that a wild-type E3-containing adenoviral vector could direct prolonged expression of a non-immunogenic transgene. Persistence of this gene expression was also attributed to the presence of the E3

25   region. Wadsworth et al. (1997) *J. Virol.* 71:5189-5196. Further, when a recombinant adenovirus vector encoding hepatitis B surface antigen and containing an intact E3 region was used to infect chimpanzees, greater viral persistence, as indicated by the duration of virus shedding, was observed compared to counterpart vectors lacking E3. This phenomenon was attributed to evasion of host immune response. Chengalvala et al. (1997)

30   *Vaccine* 15:335-339. However, the above-described E3-containing adenoviral vectors were

not replication competent and target cell specific. All of these studies employed adenovirus as a vehicle for expressing a transgene.

Use of adenoviral vectors as therapeutic vehicles for cancer has been reported. See, for example, Bischoff et al. (1996) *Science* 274:373-376; WO 96/349969; WO 96/17053. Some of these approaches utilize target cell-type specific transcriptional regulatory elements to selectively drive adenoviral replication (and thus cytotoxicity). U.S. Pat. No. 5,698,443; see also WO 95/11984; WO 96/17053; WO 98/39465; WO 98/39467; WO 98/39466; and WO 98/39464. These vectors were deleted for E3.

Based on the teachings of the prior art, inclusion of E3 is not indicated in the context of using adenoviral vector replication for its cytotoxic effects (as opposed to using an adenoviral vectors as gene delivery vehicles), as suppression of the host's cytotoxic T cell response would not be considered a positive or desirable result. Further, inclusion of E3 into replication-competent adenoviral vectors would not be indicated since the well-accepted understanding in the art is that E3 is not necessary for viral replication.

Besides cancerous cells, it is often desirable to selectively destroy certain unwanted cells or tissues. Apart from surgery, however, which is invasive, there is a dearth of methods available, particularly non-invasive methods, which would allow such selective cytotoxicity and/or suppression.

There is a need for vector constructs that are capable of rapidly eliminating cancerous cells in a minimum number of administrations and which are suitable for use in cancer ablation treatments. There is also a need for an ability to selectively destroy, or impair, unwanted cells, regardless of cell type and/or regardless of anatomical location.

All publications and patent applications cited herein are hereby incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The invention provides target cell-specific adenoviral vectors (i.e., they are preferentially cytotoxic toward a specific target cell) comprising E3 (or an E3 sequence, or a portion of an E3 region), compositions, host cells, and kits comprising these vectors, and methods using these vectors. Preferably, the vectors are replication competent. These

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target cell-specific vectors express E3-encoded protein(s) and exhibit significantly greater cytotoxicity and/or enhanced adenoviral production per cell.

Accordingly, in one aspect, the invention provides an adenovirus vector comprising (a) an adenovirus gene under transcriptional control of a target cell-specific transcriptional regulatory element (TRE); and (b) an E3 region. In another aspect, an adenovirus vector of the invention comprises (a) an adenovirus gene under transcriptional control of a target cell-specific transcriptional regulatory element (TRE); and (b) an E3 sequence. In some embodiments the adenoviral gene under transcriptional control of a target cell-specific TRE is one that is essential for adenoviral propagation, such as E1A and/or E1B. In some embodiments, the target cell-specific TRE confers cell type-specific transcriptional regulation on the operably linked adenoviral gene. In other embodiments, the target cell-specific TRE is a cell status-specific TRE.

In other embodiments, the invention provides an adenovirus vector comprising (a) an adenovirus gene under transcriptional control of a target cell-specific transcriptional regulatory element (TRE); and (b) a portion of an E3 region.

In another aspect, the invention provides a replication competent adenovirus vector comprising an E3 sequence (or E3 region) under transcriptional control of a target cell specific TRE.

In another aspect, E3-containing, target-cell-specific adenoviral vectors of the invention further contain one or more transgenes. In some of these embodiments, a transgene can be under transcriptional control of a heterologous TRE, which may be a target cell-specific TRE.

In another aspect, the invention provides an adenoviral vector comprising an E3 region (or a portion of an E3 region, or an E3 sequence) under transcriptional control of a target-cell specific TRE.

In another aspect, the invention provides a host cell comprising an adenovirus vector(s) described herein.

In another aspect, the invention provides compositions comprising an adenovirus vector(s) described herein, preferably further comprising a pharmaceutically acceptable excipient.

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In another aspect, the invention provides kits which contain an adenoviral vector(s) described herein.

In another aspect, methods are provided for propagating an adenovirus specific for mammalian cells which permit the function of a target cell-specific TRE, said method
5   comprising combining an adenovirus vector(s) described herein with mammalian cells that permit the function of a target cell-specific TRE, such that the adenovirus vector(s) enters the cell, whereby said adenovirus is propagated.

In another aspect, methods are provided for conferring selective cytotoxicity in target cells, comprising contacting the cells with an adenovirus vector(s) described herein,
10   whereby the vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, more particularly a target tumor cell, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

15   In another aspect, methods are provided for detecting a cell which allows the function of a target cell-specific TRE, which comprise contacting a cell in a biological sample with an adenovirus vector(s) of the invention, and detecting replication of the adenovirus vector(s), if any.

20   BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the E3 region of adenovirus. Letters above the boxes indicate known cellular locations of the corresponding polypeptide as follows: C, cytoplasm; ER M, endoplasmic reticulum membrane; NM, nuclear membrane; and PM, plasma membrane.

25   Figure 2 is a schematic representation of adenovirus vector CN702, which is wild-type but E3-deleted, and recombinant adenovirus vectors CN706 (lacking E3), CN739 (lacking E3), and CN787 (containing E3). With respect to adenoviral constructs (as opposed to precursor plasmid constructs), it is understood that "CN" and "CV" designations may be used interchangeably. For example, CN787 and CV787 refer to the
30   same adenoviral construct.

Figure 3 is a bar graph depicting the titer, normalized to the titer of CN702 on 293 cells, of CN702 (first bars), CN706 (second bars), CN739 (third bars), and CN787 (fourth bars) in various cell lines.

Figure 4 is a half-tone reproduction of plaques formed by CN739 and CN787 on 293 cells.

Figure 5 is a graph depicting the extracellular viral yield, expressed as plaque-forming units (PFU), from LNCaP cells at various times post-infection, of adenoviral vectors CN739 (triangles) and CN787 (squares).

Figure 6 is a graph depicting, at various times post-infection, the total virus yield, expressed as plaque-forming units (PFU), in LNCaP cells infected with CN739 (squares) or CN787 (diamonds).

Figure 7 is a graph depicting the results of an MTT assay for cytotoxicity, versus days post infection, of CN739-infected (circles), CN787-infected (squares), and mock-infected (triangles) LNCaP cells.

Figure 8 is a graph depicting viability, as measured by trypan blue exclusion, versus days post infection of LNCaP cells infected with CN739 (squares) or CN787 (circles).

Figure 9 is a graph depicting relative tumor volumes over a period of five weeks in mice with tumor xenografts. The mice were treated with CN739 (squares), CN787 (circles), or vehicle alone (triangles).

Figure 10 is a graph depicting relative tumor volumes over a period of four weeks in mice with tumor xenografts. The mice were treated with 2.5×10^{11} PFU CN787 (squares), 3×10^{11} PFU CN787 (circles), or vehicle alone (triangles).

Figure 11 is a schematic representation of adenoviral vectors CN733 and CN790.

Figure 12 is a bar graph depicting the plaquing efficiency of CN702, CN733, CN739, and CN790 in various cell lines. For each cell line tested, the first, second, third and fourth bars represent the results obtained with CN702, CN733, CN739, and CN790, respectively. A star over a bar indicates no detectable plaques.

Figure 13 is a graph depicting the relative tumor volumes over a period of six weeks in mice with HepG2 tumor xenografts. Mice were treated with 10 injections of 1×10^{10} particles per dose of CN733 (diamonds), CN739 (squares), CN790 (triangles), or vehicle alone (crosses).

Figure 14 is a graph depicting relative serum concentrations of AFP in mice with tumor xenografts, after treatment with 10 injections of 1 x 10^{10} particles per dose of CN733 (diamonds), CN739 (squares), CN790 (triangles), or vehicle alone (crosses).

Figure 15 is a schematic representation of the 5' flanking region of the carcinoembryonic antigen (CEA) gene.

Figure 16 is a schematic representation of adenoviral constructs CN802, CN799, and CN798, all of which contain E3. Specificity and extent of killing are also indicated for each virus.

Figure 17 is a bar graph depicting the number of plaque-forming units 48 hours after infection of various cell lines with CN802 (left bars), CN799 (middle bars), or CN798 (right bars).

Figure 18 is a bar graph depicting the relative viral DNA yield obtained when various cell lines were infected with CN802 (stippled bars) or CN798 (solid bars). Stars indicate no detectable virus.

Figure 19 is a schematic representation of CN796 and CN797.

Figure 20 shows the nucleotide sequence of a hypoxia-responsive element (HRE) from the 5' flanking region of a rat enolase-1 gene (SEQ ID NO:7).

Figure 21 shows the nucleotide sequence of the 5' flanking region of a human E2F1 gene (SEQ ID NO:8). The asterisk indicates the transcription start site.

Figure 22 depicts a nucleotide sequence of a PSA-TRE (SEQ ID NO:9).

Figure 23 depicts a nucleotide sequence of a CEA-TRE (SEQ ID NO:10). The bent arrow above nucleotide 14,466 indicates the transcription start site.

Figure 24 depicts a nucleotide sequence of a human glandular kallikrein TRE (SEQ ID NO:11).

Figure 25 depicts a nucleotide sequence of a mucin TRE (SEQ ID NO:12).

Figure 26 depicts a nucleotide sequence of a rat probasin TRE (SEQ ID NO:13).

Figure 27 depicts the nucleotide sequence of the AFP-TRE driving expression of E1A in CN733 (SEQ ID NO:14).

Figure 28 depicts a nucleotide sequence of an AFP-TRE (SEQ ID NO:15).

Figure 29 is a half-tone reproduction of Southern blots of viruses hybridized with a 938 bp ^{32}P-labeled E1a probe (Figure 29A), or with an 881 bp ^{32}P-labeled E1b probe (Figure 29B).

Figure 30 is a graph depicting accumulation of deletion mutants, as measured by Molecular Imager scans of Southern blot radiograms as prepared in Figure 29B. CN790 was prepared serially through 5 passages in 293 cells at MOI = 10 (square) and MOI = 0.1 (diamond), and in HepG2 cells at MOI = 10 (closed circles) and MOI = 0.1 (triangles).

Figure 31 is a half-tone reproduction of plaques formed by CN733 and CN790 on 293 cells.

Figure 32 is a bar graph depicting the virus yield of CN790 (shaded bars) and wt Ad5 (open bars, CN802) in various cell lines.

Figure 33 is a half-tone reproduction of cells stained with crystal violet comparing cytotoxicity of CN790 and CN802 in various cell lines.

Figures 34A and 34B are bar graphs depicting the relative tumor volumes (Figure 34A) and relative serum AFP concentrations (Figure 34B) in nude mice bearing Hep3B tumor xenografts. In Figure 34A, mice were treated with 3 injections of 4×10^9 pfu of CN790 (squares, n=7) or vehicle alone (triangles, n=8). Arrows indicate injection times. In Figure 34B, mice were treated with either CN790 (squares) or vehicle alone (triangles).

Figure 35 is a half-tone reproduction of immunohistochemical analysis of Hep3B xenograft tumors in nude mice treated with CN790. Adenovirus infected cells were detected by polyclonal rabbit antibody to Ad5 hexon and indicated by a filled arrow (Figure 35A). Apoptotic bodies were detected by TdT labeling and indicated by the filled arrow (Figure 35B). Photos at 400X.

MODES FOR CARRYING OUT THE INVENTION

We have discovered target cell-specific adenovirus vectors which contain an E3 region (or portion of an E3 region). In some embodiments, the adenovirus vectors of the invention contain an E3 sequence. Preferably, the adenovirus vector(s) is replication-competent, and, even more preferably, replicates preferentially in target cells. Inclusion of an E3 region (or E3 sequence, or portion of an E3 region) is contrary to the teachings of the prior art, which clearly indicated that deletion, not inclusion, of E3 would be desirable in

this context (i.e., conferring cell-specific cytotoxicity) due to E3's ability to dampen host cytotoxic response against infected cells. Moreover, the prior art teaching and emphasis that E3 is not necessary for viral replication indicates non-inclusion of E3 for replication-competent adenoviral vectors.

We have found that these vectors maintain their high level of specificity and are (a) significantly more cytotoxic; and/or (b) produce higher virus yield including extracellular virus yield; and/or (c) form larger plaques; and/or (d) produce rapid cell death; and/or (e) kill tumors or suppress tumor growth more efficiently in vivo than vectors lacking the E3 region. In addition, these vectors are target cell-specific, i.e., they comprise a target cell-specific transcriptional regulatory element, such as a cell type- or cell status-specific transcriptional regulatory element(s) (TRE), that preferably drive an adenovirus gene essential for propagation, preferably one or more early genes. The vectors of the invention are useful for exerting selective cytotoxicity and/or suppressing cell growth (including tumor growth). This is especially useful in the cancer context, in which targeted cell killing is desirable. This is also useful for targeted cytotoxic effects in other, non-tumor cells, when selective destruction and/or suppression of these cells is desirable. The vectors can also be useful for detecting the presence of cells which permit function of a target cell-specific TRE in, for example, an appropriate biological (such as clinical) sample. Further, the adenovirus vector(s) can optionally selectively produce one or more proteins of interest in a target cell by using a heterologous, i.e., non-adenoviral, TRE.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The

Polymerase Chain Reaction", (Mullis et al., eds., 1994); "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

For techniques related to adenovirus, see, *inter alia*, Felgner and Ringold (1989) *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham 5 (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

Definitions

An "E3 region" (used interchangeably with "intact E3 region") is a term well understood in the art and means the region of the adenoviral genome that encodes the E3 10 products (discussed herein). Generally, the E3 region is located between about 28583 and about 30470 of the adenoviral genome. An E3 region (or E3 sequence, defined below) for use in the present invention may be from any adenovirus serotype. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274.

15 An "E3 sequence" is a polynucleotide sequence that contains a sequence from an E3 region and excludes a sequence encoding only ADP. Thus, an E3 sequence can include ADP, as long as another portion of the E3 region is included. As is well known in the art, the ADP coding region is located in the E3 region within the adenoviral genome from about 29468 bp to about 29773 bp; including the Y leader, the location of ADP is from 20 about 28375 bp to about 29773 bp for Ad5. Other ADP regions for other serotypes are known in the art. An E3 sequence includes, but is not limited to, deletions; insertions; fusions; and substitutions. An E3 sequence may also comprise an E3 region or a portion of the E3 region. It is understood that, as an "E3 sequence" is not limited to an "E3 region", alternative references herein to an "E3 region" or "E3 sequence" do not indicate that these 25 terms are interchangeable. Assays for determining a functional E3 sequence for purposes of this invention are described herein.

An "E3 containing" vector of the invention refers to any of the embodiments described herein.

A "portion" of the E3 region means less than the entire E3 region, and as such 30 includes polynucleotide deletions as well as polynucleotides encoding one or more polypeptide products of the E3 region. For purposes of this invention, when a "portion" of

E3 region includes a polynucleotide encoding ADP, that portion includes at least one other E3 polypeptide product, or a functional fragment of an E3 polypeptide product.

As used herein, a "transcriptional regulatory element", or "TRE" is a polynucleotide sequence, preferably a DNA sequence, that regulates (i.e., controls) transcription of an operably-linked polynucleotide sequence by an RNA polymerase to form RNA. As used herein, a TRE increases transcription of an operably linked polynucleotide sequence in a host cell that allows the TRE to function. The TRE comprises an enhancer element and/or promoter element, which may or may not be derived from the same gene. The promoter and enhancer components of a TRE may be in any orientation and/or distance from the coding sequence of interest, and comprise multimers of the foregoing, as long as the desired transcriptional activity is obtained. As discussed herein, a TRE may or may not lack a silencer element.

As used herein, the term "target cell-specific TRE" is intended to mean that the TRE sequences to which a gene, which may be a gene essential for replication of an adenoviral vector, is operably linked, or to which a transgene is operably linked, functions specifically in that target cell so that transcription (and replication, if the operably linked gene is one essential for adenovirus replication) selectively or preferentially proceeds (i.e., when compared to a non-target cell) in that target cell, or so that a transgene polynucleotide is expressed in that target cell. This can occur by virtue of the presence in that target cell, and not in non-target cells (or significantly less so, or in inactive or less active forms, in non-target cells), of transcription factors that activate transcription driven by the operably linked transcriptional control sequences. It can also occur by virtue of the absence of transcription inhibiting factors that normally occur in non-target cells and prevent transcription driven by the operably linked transcriptional control sequences. "Target cell-specific TRE" includes cell type-specific and cell status-specific TRE, as well as "composite" TREs. The term "composite TRE" includes a TRE which comprises both a cell type-specific and a cell status-specific TRE. A target cell-specific TRE can also include a heterologous component, including, for example, an SV40 or a cytomegalovirus (CMV) promoter(s).

A "cell type-specific TRE" is preferentially functional, i.e., confers transcriptional activation, in a specific type of cell relative to other types of cells of different functionality.

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"Cell type" is a reflection of a differentiation state of a cell which is, under normal physiological conditions, an irreversible, end-stage state. For example, a prostate-specific antigen TRE is functional in prostate cells, but is not substantially, or significantly, functional in other cell types such as hepatocytes, astrocytes, cardiocytes, lymphocytes, etc.

5   Generally, a cell type-specific TRE is active in only one cell type. When a cell type-specific TRE is active in more than one cell type, its activity is restricted to a limited number of cell types, i.e., it is not active in all cell types. A cell type-specific TRE may or may not be tumor cell specific. The term "cell type-specific", as used herein, is intended to include cell type specificity, tissue specificity, as well as specificity for a cancerous state of

10   a given target cell type. In the latter case, specificity for a cancerous state of a normal cell is in comparison to a normal, non-cancerous counterpart.

As used herein, the term "cell status-specific TRE" is preferentially functional, i.e., confers transcriptional activation on an operably linked polynucleotide in a cell which · allows a cell status-specific TRE to function, i.e., a cell which exhibits a particular

15   physiological condition, including, but not limited to, an aberrant physiological state. "Cell status" thus refers to a given, or particular, physiological state (or condition) of a cell, which is reversible and/or progressive. The physiological state may be generated internally or externally; for example, it may be a metabolic state (such as in response to conditions of low oxygen), or it may be generated due to heat or ionizing radiation. "Cell status" is

20   distinct from a "cell type", which relates to a differentiation state of a cell, which under normal conditions is irreversible. Generally (but not necessarily), as discussed herein, a cell status is embodied in an aberrant physiological state, examples of which are given below.

A "normal cell status" or "normal physiological state" is the status of a cell which

25   exists in normal physiological conditions and which is non-dividing or divides in a regulated manner, i.e., a cell in a normal physiological state.

"Normal physiological conditions" are known to those skilled in the art. These conditions may vary, depending on a cell's location in the body. For example, oxygen tension can vary from tissue to tissue. For *in vitro* analyses for the purposes of determining

30   whether a TRE is responsive to deviations from normal physiological conditions, these conditions generally include an oxygen concentration of about 20% O_2, and a temperature

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of about 37°C. "Regulated cell division" is a term well understood in the art and refers to the normal mitotic activity of a cell. Those skilled in the art understand that normal mitotic activity varies from cell type to cell type. For example, many terminally differentiated cells in tissues exhibit little or no mitotic activity, while hematopoietic cells are generally

5   mitotically active.

The terms "aberrant cell status" and "aberrant physiological state", used interchangeably herein, intend a condition of a cell which is a response to, a result of, or is influenced by, an aberrant physiological condition. An aberrant cell status is neither cell type-specific nor tissue type-specific. An aberrant cell status is defined in relation to a cell

10   of the same type which is in a non-dividing/regulated dividing state and under normal physiological conditions. An "aberrant physiological condition" or "aberrant physiological state", as used herein, intends a condition which deviates from normal physiological conditions, and includes, but is not limited to, a physiological condition that is characterized by alterations in oxygen concentration, such as hypoxic conditions;

15   temperatures which deviate from physiological temperatures; a condition that triggers apoptosis; radiation, including ionizing radiation and UV irradiation; de-regulated cell division, resulting for example, from a lack of, or insufficient amounts of, or inactivity of, a factor which controls cell division, such as, for example, retinoblastoma protein (Rb); variations in timing of cell cycle; infection with a pathogen; exposure to a chemical

20   substance; or a combination of the above-listed conditions. Another example is a mutation that could, or does, exist in any cell type, i.e., its existence does not depend on, or is not related to, the differentiation state of the cell.

A "target cell", as used herein, is one that allows, permits or induces the function of a target cell-specific TRE such that it effects transcriptional activation (and/or

25   enhancement), i.e., increases the level of transcription, of an operably linked polynucleotide. Target cell-specific TREs include cell type-specific TREs and cell status-specific TREs. Preferably, a target cell is a mammalian cell, preferably a human cell. A target cell may or may not be neoplastic.

"A target cell type which allows a TRE to function" or a cell in which the function

30   of a TRE is "sufficiently preserved" or "functionally preserved", or "a cell in which a TRE is functional" is a cell in which the TRE, when operably linked to a promoter (if not

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included in the TRE) and a reporter gene, increases expression of the reporter gene at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least

5   about 400- to about 500-fold, even more preferably at least about 1000-fold, when compared to the expression of the same reporter gene when not operably linked to said TRE. In the context of cell type-specific TREs, a "target cell" is a cell type which allows the function of a cell type-specific TRE. Accordingly, in the context of a cell type-specific TRE, comparison is further made between expression of the reporter gene when operably

10   linked to the cell type-specific TRE when in a target cell type versus when in a different cell type. In the context of cell status-specific TREs, a "target cell" is one which exhibits a given requisite physiological (or environmental) state, which may be an aberrant physiological state. Accordingly, in the context of a cell status-specific TRE, comparison is further made between expression of the reporter when operably linked to a cell status-

15   specific TRE in the target cell in a given physiological state (which may be an aberrant physiological state) versus the same cell when in a normal physiological state (or a physiological state different from the given physiological state). Methods for measuring levels (whether relative or absolute) of expression are known in the art and are described herein.

20   A "functionally-preserved variant" of a target cell-specific TRE is a target cell-specific TRE which differs from another target cell-specific TRE, but still retains target cell-specific transcription activity, although the degree of activation may be altered (as discussed below). The difference in a target cell-specific TRE can be due to differences in linear sequence, arising from, for example, single base mutation(s), addition(s), deletion(s),

25   and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a target cell-specific TRE.

As used herein, a TRE derived from a specific gene is referred to by the gene from which it was derived and is a polynucleotide sequence which regulates transcription of an operably linked polynucleotide sequence in a host cell that expresses said gene. For

30   example, as used herein, a "human glandular kallikrein transcriptional regulatory element", or "*hKLK2*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which

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increases transcription of an operably linked polynucleotide sequence in a host cell that allows an *hKLK2*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor, such as a prostate cell. An *hKLK2*-TRE is thus responsive to the binding of androgen receptor and comprises at least a

5   portion of an *hKLK2* promoter and/or an *hKLK2* enhancer (i.e., the ARE or androgen receptor binding site).

     As used herein, a "probasin (*PB*) transcriptional regulatory element", or "*PB*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably-linked polynucleotide sequence in a host cell that allows a *PB*-

10   TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PB*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PB* promoter and/or a *PB* enhancer (i.e., the ARE or androgen receptor binding site).

     As used herein, a "prostate-specific antigen (*PSA*) transcriptional regulatory

15   element", or "*PSA*-TRE", or "*PSE*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *PSA*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PSA*-TRE is thus responsive to the binding of androgen

20   receptor and comprises at least a portion of a *PSA* promoter and/or a *PSA* enhancer (i.e., the ARE or androgen receptor binding site).

     As used herein, a "carcinoembryonic antigen (*CEA*) transcriptional regulatory element", or "*CEA*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host

25   cell that allows a *CEA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses CEA. The *CEA*-TRE is responsive to transcription factors and/or co-factor(s) associated with CEA-producing cells and comprises at least a portion of the *CEA* promoter and/or enhancer.

     As used herein, an "α-fetoprotein (*AFP*) transcriptional regulatory element", or

30   "*AFP*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably linked polynucleotide sequence) in a host cell that

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allows an *AFP*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses AFP. The *AFP*-TRE is responsive to transcription factors and/or co-factor(s) associated with AFP-producing cells and comprises at least a portion of the *AFP* promoter and/or enhancer.

5   As used herein, an "a mucin gene (*MUC*) transcriptional regulatory element", or "*MUC1*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably-linked polynucleotide sequence) in a host cell that allows a *MUC1*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses MUC1. The *MUC1*-TRE is responsive to

10   transcription factors and/or co-factor(s) associated with MUC1-producing cells and comprises at least a portion of the *MUC1* promoter and/or enhancer.

An "adenovirus vector" or "adenoviral vector" (used interchangeably) comprises a polynucleotide construct of the invention. A polynucleotide construct of this invention may be in any of several forms, including, but not limited to, DNA, DNA encapsulated in

15   an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed with synthetic polycationic molecules, conjugated with transferrin, and complexed with compounds such as PEG to immunologically "mask" the molecule and/or increase half-life, and conjugated to a nonviral protein. Preferably, the polynucleotide is

20   DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

25   The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or

30   derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted

sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH2) or a mixed phosphoramidate-phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8;

5   Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothiate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995) *Mol. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by

10   synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant

15   polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-

20   nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support.

25   A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular*

30   *Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software,

Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; and extend gap = 2. Another preferred program is the BLAST program for alignment of two nucleotide sequences, using default parameters as follows: open gap = 5; extension gap - 2 penalties; gap x dropoff = 50; expect = 10; word size = 11. The BLAST program is available at the following Internet address: http://www.ncbi.nlm.nih.gov.

A "replication competent" adenoviral vector or adenovirus is a term well-understood in the art and means that the adenoviral vector is able to replicate, or propagate. "Replication" and "propagation" are defined herein.

"Under transcriptional control" is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. "Operably linked" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

As used herein, "cytotoxicity" is a term well understood in the art and refers to a state in which a cell's usual biochemical or biological activities are compromised (i.e., inhibited). These activities include, but are not limited to, metabolism; cellular replication; DNA replication; transcription; translation; uptake of molecules. "Cytotoxicity" includes cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, ^{3}H-thymidine uptake, and plaque assays.

The term "selective cytotoxicity", as used herein, refers to the cytotoxicity conferred by an adenovirus vector of the present invention on a cell which allows or induces a target cell-specific TRE to function (a target cell) when compared to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which does not allow a target cell-specific TRE to function (a non-target cell). Such cytotoxicity may be measured, for example, by plaque assays, by reduction or stabilization in size of a tumor comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells, or a tissue-specific marker, e.g., a cancer marker, such as prostate specific antigen.

In the context of adenovirus or adenovirus vector, a "heterologous polynucleotide" or "heterologous gene" or "transgene" (used interchangeably) is any polynucleotide or gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be

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expressed or present in the target cell prior to introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

In the context of adenovirus or adenovirus vector, a "heterologous" promoter or enhancer or TRE is one which is not associated with or derived from an adenovirus gene.

5   In the context of adenovirus or adenovirus vector, a "heterologous target cell-specific TRE" is a target cell-specific TRE which is not associated with or derived from an adenovirus gene.

In the context of adenovirus or adenovirus vector, an "endogenous" promoter, enhancer, or TRE is native to or derived from adenovirus.

10   In the context of a target cell-specific TRE, a "heterologous" promoter or enhancer is one which is not normally associated in a cell with or derived from the target cell-specific TRE. Examples of a heterologous promoter or enhancer are the albumin promoter or enhancer and other viral promoters and enhancers, such as SV40 or CMV, or cell type specific TREs such as a prostate specific TRE (for example, a prostate specific TRE is

15   heterologous with respect to a liver cell specific TRE).

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are

20   proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively

25   autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector(s) of this invention. Host cells include progeny of a single

30   host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate

mutation and/or change. A host cell includes cells transfected or infected *in vivo* or *in vitro* with an adenoviral vector of this invention.

"Replication" and "propagation" are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. These terms are well

5   understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay, plaque assay, or a one-step growth curve. "Replication" and "propagation" include any activity directly or indirectly involved in the process of virus manufacture, including,

10   but not limited to, viral gene expression; production of viral proteins, nucleic acids or other components; packaging of viral components into complete viruses; and cell lysis.

"Replicating preferentially" means that the adenovirus replicates more in a target cell than in a non-target cell. Preferably, the adenovirus replicates at least about 10%, more preferably at least about 25%, more preferably at least about 50%, more preferably at least

15   about 100% (i.e., 2-fold) higher, preferably at least about 5-fold higher, more preferably at least about 10-fold higher, still more preferably at least about 50-fold higher, even more preferably at least about 100-fold higher, still more preferably at least about 400-fold to about 500-fold higher, still more preferably at least about 1000-fold higher, most preferably at least about 1×10^6 higher.

20   A polynucleotide sequence that is "depicted in" a SEQ ID NO means that the sequence is present as, or contained within an identical contiguous sequence in the SEQ ID NO. The term encompasses portions, or regions of the SEQ ID NO as well as the entire sequence contained within the SEQ ID NO.

A "biological sample" encompasses a variety of sample types obtained from an

25   individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain

30   components, such as proteins or polynucleotides. The term "biological sample"

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encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, rodents, primates,

5   and pets.

An "effective amount" is an amount sufficient to effect beneficial or desired results, including clinical results. An effective amount can be administered in one or more administrations. In the context of a disease state, an effective amount of an adenoviral vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay

10   the progression of the disease state.

E3-containing target cell-specific adenoviral vectors

The invention provides target cell-specific adenovirus vectors comprising an E3 sequence and an adenoviral gene under transcriptional control of a target cell-specific TRE. Preferably, the vectors are replication-competent. Even more preferably, the adenoviral

15   gene under transcriptional control of a target cell-specific TRE is one essential for adenoviral replication. Inclusion of the E3 region of adenovirus was found to enhance cytotoxicity of the target cell-specific adenoviral vectors of the present invention.

E3-containing adenoviral vectors of the invention (including adenoviral vectors containing E3 sequences and/or a portion of E3) maintain their high level of specificity and

20   display one or more of the following characteristics relative to adenoviral vectors lacking an E3 region (or portion of E3), i.e., they (a) are more cytotoxic; (b) produce higher virus yield, including intracellular and extracellular virus yield; (c) form larger plaques; (d) produce rapid cell death; (e) kill tumors more efficiently *in vivo*; and/or (f) suppress tumor growth more efficiently *in vivo*. Any detectable increase is sufficient, although preferably

25   any of these characteristics may be increased (when compared to adenoviral vectors lacking E3) at least about 10%, more preferably at least about 25%, more preferably at least about 50%, more preferably at least about 100% (i.e., 2-fold), more preferably at least about 5-fold, more preferably at least about 10-fold, more preferably at least about 20-fold or more. In some embodiments, cytotoxicity of an E3-containing adenoviral vector of the invention

30   is increased when compared to the cytotoxicity of an adenoviral counterpart lacking E3. It is understood that an E3-containing adenoviral vector of the invention can exhibit the

aforementioned increased level(s) of activity with respect to one or more of these characteristics, to the same or different degree relative to an adenoviral counterpart lacking E3. For example, a given E3-containing adenoviral vector may exhibit 2-fold greater cytotoxicity, yet produce a 3-fold higher virus yield than an adenoviral counterpart lacking

5   E3.

Cytotoxicity can be measured by any known method, including, but not limited to, an MTT assay and trypan blue dye exclusion, both of which are described in Example 2. Virus yield can be measured by any known method, including, but not limited to, plaque assay on a cell line permissive for adenoviral replication, as described in Example 2.

10   Whether a given E3-containing adenovirus vector forms larger plaques than a counterpart vector lacking E3 can be determined, for example, by plaque assay and measuring plaque diameter. Whether a given E3-containing adenovirus vector produces rapid cell death relative to a counterpart adenovirus vector lacking E3 can be determined using any known method, including, but not limited to, MTT assay or trypan blue exclusion assay performed ·

15   over a suitable course of time post-infection, such as 1, 2, 3, 5, or 7 or more days post-infection. Tumor killing and suppression of tumor cell growth can be measured by any known method, including, but not limited to, a tumor xenograft assay as described in Example 2.

It is understood that, for purposes of this invention, not only may an (intact) E3

20   region or a portion of an E3 region be used, but E3 sequences (which include other forms and arrangements of E3) may be employed, including, but not limited to, deletions; internal deletions; fusions comprising E3 or a portion of E3; insertions (whereby one or more nucleotides are inserted within an E3 sequence); and substitutions, including silent substitutions, as well as substitutions resulting in one or more differences in an amino acid

25   sequence(s). A functional E3 squence may be determined by assaying, for example, for any of the characteristics listed above.

The adenoviral vectors of this invention contain an E3 sequence, which may be an intact E3 region or a portion of an E3 region. It is understood that, as inclusion of E3 confers observable and measurable functionality on the adenoviral vectors, for example,

30   increased replication and production, functionally equivalent (in which functionality is essentially maintained, preserved, or even enhanced or diminished) variants of E3 may be

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constructed. Functional equivalents of an E3 region include those in which one or more E3 functions is essentially maintained, preserved, or even enhanced or diminished which confer one or more of the following properties on the virus: (a) cytotoxicity; (b) production of virus yield, including intracellular and extracellular virus yield; (c) plaque formation;

5   and (d) production of cell death; (e) killing and/or suppressing of tumors *in vivo*. For example, portions of E3 may be used. As is explained in the definition of "portion" of E3, a portion may be, non-inclusively, either of the following: (a) deletion, preferably at the 3' end; (b) inclusion of one or more various open reading frames of E3. Five proteins which are encoded by the Ad-E3 region have been identified and characterized: (1) a 19-kDa

10   glycoprotein (gp19k) is one of the most abundant adenovirus early proteins, and is known to inhibit transport of the major histocompatibility complex class I molecules to the cell surface, thus impairing both peptide recognition and clearance of Ad-infected cells by cytotoxic T lymphocytes (CTLs); (2) E3 14.7k protein and the E3 10.4k/14.5k complex of proteins inhibit the cytotoxic and inflammatory responses mediated by tumor necrosis

15   factor (TNF); (3) E3 10.4k/14.5k protein complex downregulates the epidermal growth factor receptor, which may inhibit inflammation and activate quiescent infected cells for efficient virus replication; (4) E3 11.6k protein (adenoviral death protein, ADP) from adenovirus 2 and 5 appears to promote cell death and release of virus from infected cells. The functions of three E3-encoded proteins -- 3.6k, 6.7k and 12.5k -- are unknown. A

20   ninth protein having a molecular weight of 7.5 kDa has been postulated to exist, but has not been detected in cells infected with wild-type adenovirus. Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. The E3 region is schematically depicted in Figure 1. These intact, portions, or variants of E3 may be readily constructed using standard knowledge and techniques in the art. Preferably, an intact E3 region is used. Appropriate

25   vectors comprising an intact E3 region are commercially available, e.g., from Microbix. In other embodiments, an E3 region is used which lacks the 10.4k/14.5k complex and the 14.7 protein (i.e., deletion of about bp 29781 to about bp 30847). E3 sequences or portions of E3 can be derived from such vectors using standard techniques of molecular biology, such as restriction and ligation, and polymerase chain reactions. Any of the various serotypes of

30   adenovirus can be used in the present invention, such as Ad2, Ad5, Ad12 and Ad40. For purposes of illustration, serotype Ad5 is exemplified herein.

In the adenovirus vectors of the present invention, E3 (i.e., E3 sequence, which includes E3 region or portion of an E3 region) may or may not be under transcriptional control of native adenoviral transcriptional control element(s). The E3 promoter is located within the coding sequence for virion protein VIII, an essential protein which is highly

5   conserved among adenovirus serotypes. In some embodiments, E3 is under transcriptional control of a heterologous TRE, including, but not limited to, a target cell-specific TRE. In one embodiment, the invention provides an adenoviral vector, preferably replication competent, that comprises an E3 sequence under transcriptional control of a target-cell specific TRE. A number of target cell-specific TREs are known in the art, several of which

10   are described in more detail herein. In other embodiments, the E3 region is under transcriptional control of a target cell-specific TRE, and the vector further comprises an adenoviral gene essential for replication under transcriptional control of a target cell-specific TRE.

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Target cell-specific TREs

Adenoviral vectors of the present invention include, in addition to an E3 sequence, one or more adenoviral genes under transcriptional control of a target cell-specific TRE, and optionally include a transgene, which may also be under transcriptional control of a

20   target cell-specific TRE or other TRE. Preferably, the adenovirus gene(s) under transcriptional control of a target cell-specific TRE is essential for propagation, preferably an early gene(s), such as E1A, E1B, and/or E4. When the adenovirus vector(s) is selectively (i.e., preferentially) replication competent for propagation in target cells, these cells will be preferentially killed upon adenoviral proliferation. Once the target cells are

25   destroyed due to selective cytotoxic and/or cytolytic replication, the adenovirus vector replication is significantly reduced, thus lessening the probability of runaway infection and undesirable bystander effects. *In vitro* cultures may be retained to monitor the mixture (such as, for example, a biopsy or other appropriate biological sample) for occurrence (i.e., presence) and/or recurrence of the target cell, e.g., a neoplastic cell or other undesired cell.

30   To further ensure cytotoxicity, one or more transgenes having a cytotoxic effect may also be present and under selective transcriptional control. In this embodiment, one may

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provide higher confidence that the target cells will be destroyed. Target cell-specific TREs include cell type-specific TREs and cell status-specific TREs, either of which may be specific for a neoplastic cell. Alternatively, an adenovirus gene, and optionally, a transgene, can be under transcriptional control of a cell type-specific and a cell status-
5   specific TRE. Such target cell-specific TREs are termed "composite" target cell-specific TREs herein.

Target cell-specific TREs for use in the adenoviral vectors of the present invention can be derived from any species, preferably a mammal. A number of genes have been described which are expressed preferentially in specific cell types. Any of these cell type-
10   specific genes can be used to generate a cell type-specific TRE. Similarly, a number of genes have been described which are expressed in response to, or in association with, a cell status. Any of these cell status-associated genes may be used to generate a cell status-specific TRE.

A target cell-specific TRE may or may not lack a silencer. The presence of a
15   silencer (i.e., a negative regulatory element) may assist in shutting off transcription (and thus replication) in non-permissive cells (i.e., cell in a normal cell state). Thus, presence of a silencer may confer enhanced target cell-specific replication by more effectively preventing adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in effecting replication in target cells, thus conferring enhanced target
20   cell-specific replication due to more effective replication in target cells.

A target cell-specific TRE can comprise multimers, for example, a tandem series of at least two, at least three, at least four, or at least five target cell-specific response elements. These multimers may also contain heterologous promoter and/or enhancer sequences.
25   In some embodiments, an E3-containing adenoviral vector of the invention further comprises a first adenoviral gene under transcriptional control of a first target cell-specific TRE and a second gene under transcriptional control of a second target cell-specific TRE. In some of these embodiments, the first adenoviral gene is one essential for adenoviral replication. In other embodiments, the first and the second genes are adenoviral genes. In
30   other embodiments, the first and the second genes are adenoviral genes essential for replication.

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The first and the second target cell-specific TREs may or may not be substantially identical to one another. By "substantially identical" is meant a requisite degree of sequence identity between the two TREs. The degree of sequence identity between these TREs is at least about 80%, preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 98%, and most preferably 100%.

Adenoviral constructs in which the first and second target cell-specific TREs are substantially identical, particularly if these TREs control transcription of early genes (such as E1A and E1B), may display an instability which may be desirable in certain contexts, such as when an automatic "self-destruction" property can shut down the virus, thereby controlling the degree of propagation. Conversely, more stable double (or more) constructs may be desirable in yet other contexts. In either case, we have observed that having multiple adenovirus genes essential for propagation (especially early genes, more particularly E1a and E1b), confers significant specificity to the vectors. In some embodiments, the first and second target cell-specific TREs are cell type-specific and are functional in the same cell type.

Sequence identity can be determined by a sequence comparison using, i.e., sequence alignment programs that are known in the art, such as those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1 A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; and extend gap = 2. Another preferred program is the BLAST program for alignment of two nucleotide sequences, using default parameters as follows: open gap = 5; extension gap - 2 penalties; gap x dropoff = 50; expect = 10; word size = 11. The BLAST program is available at the following Internet address: http://www.ncbi.nlm.nih.gov. Alternatively, hybridization under stringent conditions can also indicate degree of sequence identity.

Hybridization reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridization reaction of widely known and published in the art. See, for example, Sambrook et al. (1989). Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of

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25°C, 37°C, 50°C and 68°C; buffer concentrations of 10 X SSC, 6 X SSC, 1 X SSC, 0.1 X SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1,

5   2, or 15 minutes; and wash solutions of 6 X SSC, 1 X SSC, 0.1 X SSC, or deionized water.

"T_m" is the temperature in degrees Celsius at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in anti-parallel direction by Watson-Crick base pairing dissociates into single strands under conditions of the experiment. T_m may be predicted according to a standard formula, such as:

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$$T_m = 81.5 + 16.6 \log[X^+] + 0.41 \, (\%G/C) - 0.61 \, (\%F) - 600/L$$

where $[X^+]$ is the cation concentration (usually sodium ion, Na^+) in mol/L; (%G/C) is the number of G and C residues as a percentage of total residues in the duplex; (%F) is

15   the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

Stringent conditions for both DNA/DNA and DNA/RNA hybridization are as described by Sambrook et al. *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, herein incorporated by

20   reference. For example, see page 7.52 of Sambrook et al. An example of stringent hybridization conditions are 68°C, 0.2 x SSC.

Accordingly, in some embodiments, the invention provides an adenoviral vector comprising, in addition to an E3 sequence, any of the following: (a) E1A under transcriptional control of a *PB*-TRE and E1B under transcriptional control of a *PSA*-TRE;

25   (b) E1A under transcriptional control of an *AFP*-TRE and E1B under transcriptional control of a second (but not necessarily identical or non-identical) *AFP*-TRE; (c) E1A under transcriptional control of a *CEA*-TRE. As the discussion of possible TREs below makes clear, these are non-limiting examples of the possible combinations for the E3-containing vectors of the invention.

30   An example of an adenoviral vector comprising, in addition to an E3 sequence, E1A under transcriptional control of a *PB*-TRE and E1B under transcriptional control of a *PSA*-TRE is the construct CN787, the construction of which is described in detail in Example 1.

An example of an adenoviral vector comprising, in addition to an E3 sequence, E1A under transcriptional control of an *AFP*-TRE and E1B under transcriptional control of a second (but not necessarily identical or non-identical) *AFP*-TRE is the construct CN790, which is described in Example 1. Examples of an adenoviral vector comprising, in addition to an E3 sequence, E1A under transcriptional control of a *CEA*-TRE are CN798 and CN799, which are described in detail in Example 1. Accordingly, the present invention encompasses, as specific embodiments, CN787, CN790, CN798, and CN799 (alternatively and interchangeably denoted CV787, CV790, CV798, and CV799).

In other embodiments, the adenoviral vector comprises an adenoviral gene essential for adenoviral replication under control of a first target cell-specific TRE, and a transgene under control of a second target cell-specific TRE. The first and the second target cell-specific TREs may or may not be substantially identical to one another.

In some embodiments, a target cell-specific TRE can comprise two or more different types of TREs. For example, a cell status-specific TRE can be juxtaposed with another TRE, such as a different cell status-specific TRE, or, alternatively, a cell type-specific TRE. "Juxtaposed" means a cell status-specific TRE and the second TRE transcriptionally control the same gene. For these embodiments, the cell status-specific TRE and the second TRE may be in any of a number of configurations, including, but not limited to, (a) next to each other (i.e., abutting); (b) both 5' to the gene that is transcriptionally controlled (i.e., may have intervening sequences between them); (c) one TRE 5' and the other TRE 3' to the gene. For example, as described in Example 1 and shown in Figure 19, a cell type-specific TRE can be juxtaposed with a cell status-specific TRE to control transcription of an operably linked adenoviral gene. The construct CN797 is an example of an E3-containing adenoviral vector comprising the composite TRE described in Example 1. Accordingly, the present invention encompasses, as one embodiment, CN797 (alternatively and interchangeably denoted CV797). Such "composite" TREs can be used to confer both cell status- and cell type-specific expression of an operably linked polynucleotide, and thus may confer significantly greater specificity and/or efficacy. Examples of cell type-specific TREs are provided below. Alternatively, "composite" TREs can be used to confer different, and possibly synergistic, cell status and/or cell type specificity. For example, a composite cell status-specific TRE could

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confer specificity to hypoxia and heat shock. Example 1 provides a description of an E3-containing adenovirus construct comprising E1A and E1B genes under transcriptional control of a combination transcriptional regulatory element consisting of a hypoxia-responsive element (HRE) and a prostate-specific TRE, PSA-TRE. Accordingly, in one

5   embodiment, an HRE comprises a 67-base fragment depicted in Figure 20 (SEQ ID NO:7).

As is readily appreciated by one skilled in the art, a target cell-specific TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily available functional assays (such as the CAT or luciferase reporter gene

10   assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite target cell-specific transcription function. Hence, the invention also includes functionally-preserved variants of the nucleic acid sequences disclosed herein, which include nucleic acid substitutions, additions, and/or deletions. While not wishing to be bound by a single theory, the inventors note that it is possible that certain modifications

15   will result in modulated resultant expression levels, including enhanced expression levels. Achievement of modulated resultant expression levels, preferably enhanced expression levels, may be especially desirable in the case of certain, more aggressive forms of cancer, or when a more rapid and/or aggressive pattern of cell killing is warranted (due to an immunocompromised condition of the individual, for example).

20   The activity of a TRE generally depends upon the presence of transcriptional regulatory factors and/or the absence of transcriptional regulatory inhibitors. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operatively

25   linked to) the TRE. As discussed herein, a TRE can be of varying lengths, and of varying sequence composition.

Certain point mutations within sequences of TREs have been shown to decrease transcription factor binding and gene activation. One of skill in the art would recognize that some alterations of bases in and around known the transcription factor binding sites are

30   more likely to negatively affect gene activation and cell-specificity, while alterations in bases which are not involved in transcription factor binding are not as likely to have such

effects. Certain mutations are also capable of increasing TRE activity. Testing of the effects of altering bases may be performed *in vitro* or *in vivo* by any method known in the art, such as mobility shift assays, or transfecting vectors containing these alterations in TRE functional and TRE non-functional cells. Additionally, one of skill in the art would

5   recognize that point mutations and deletions can be made to a TRE sequence without altering the ability of the sequence to regulate transcription.

As an example of how target cell-specific TRE activity can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant

10   methods. The sequence(s) to be tested is inserted into a vector containing an appropriate reporter gene, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-galactosidase (encoded by the lacZ gene), luciferase (encoded by the luc gene), green fluorescent protein, alkaline phosphatase, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus

15   constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative target cell-specific TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE–dextran. Suitable host cells include any cell type, including but not limited to, Hep3B, Hep G2, HuH7, HuH1/C12, LNCaP, HBL-100, Chang liver cells,

20   MCF-7, HLF, HLE, 3T3, HUVEC, and HeLa. For a TRE suspected to be a cell status-specific TRE, host cells transfected with the TRE-reporter gene construct to be tested are subjected to conditions which result in a change in cell status (for example, one which result in an aberrant physiological state). The same cells not subjected to these conditions, i.e., which are under normal physiological conditions and therefore in a normal

25   physiological state, serve as controls. Results are obtained by measuring the level of expression of the reporter gene using standard assays. Comparison of expression between cells in a particular state and control indicates presence or absence of transcriptional activation. Generally, to determine cell specific activity of a TRE, the TRE-reporter gene constructs are introduced into a variety of cell types. The amount of TRE activity is

30   determined in each cell type and compared to that of a reporter gene construct without the

TRE. A TRE is cell specific when it is preferentially functional in a specific type of cell over a different type of cell. "Transcriptional activation" has been defined above.

Comparisons between or among various target cell-specific TREs can be assessed, for example, by measuring and comparing levels of expression within a single cell line

5   under normal and aberrant physiological conditions (for cell status-specific TREs) or within a single cell line of the appropriate cell type (for cell type-specific TREs). These comparisons may also be made by measuring and comparing levels of expression within a single cell line. It is understood that absolute transcriptional activity of a target cell-specific TRE will depend on several factors, such as the nature of the target cell, delivery

10   mode and form of the target cell-specific TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection

15   efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the cytomegalovirus (CMV) immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

As an example of how hypoxia induction can be measured, one can use an assay such as that described in Jiang et al. (1997) *Cancer Research* 57:5328-5335 or Dachs et al.

20   (1997) *Nature Med.* 3:515-520. For example, a construct comprising a putative HRE, or multiple tandem copies thereof, together with a minimal promoter element, operably linked and controlling transcription of a polynucleotide which encodes a protein which is detectable or can be used to give a detectable signal, is introduced into host cells. The host cells are then subjected to conditions of normoxia (e.g., 20% O_2), and varying degrees of

25   hypoxia, such as 5%, 2%, 1%, 0.1%, or less, O_2. The expression product of the operably linked polynucleotide (reporter gene) is then measured.

As an example of how a prostate cell-specific TRE activity, such as a *PSA*-TRE, can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis,

30   PCR, and/or recombinant methods. The sequence(s) to be tested can be inserted into a vector containing an appropriate reporter gene encoding a reporter protein, as described

above. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative PSA-TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE dextran. Suitable host cells include any cell type that produces androgen receptor, including but not limited to, prostate cells, including prostate tumor cells such as LNCaP. A gene encoding androgen receptor can be transformed into and expressed in any cell that does not normally express AR; in such a cell, a PSA-TRE will be functional. Non-androgen receptor producing cells, such as HLF, HLE, and 3T3 and the non-AR-producing prostate cancer cells PC3 and DU145 can be used as a control. Results are obtained by measuring the level of expression of the reporter gene using standard assays. The comparison of reporter gene expression between AR-producing cells and the control cell indicates the presence, absence, and/or degree of transcriptional activation.

Alternatively a putative cell status-specific TRE can be assessed for its ability to confer adenoviral replication preference for cells exhibiting the requisite physiological state, such as heat or ionizing radiation. For this assay, constructs containing an adenovirus gene essential to replication operably linked to a putative cell status-specific TRE are transfected into cells which exhibit the requisite physiological state. Viral replication in those cells is compared, for example, to viral replication by the construct in cells under normal physiological conditions (i.e., not exhibiting the requisite physiological state).

When a target cell-specific TRE is used with an adenovirus gene that is essential for propagation replication, competence is preferentially achievable in the target cell type and/or the target cell exhibiting a particular cell status.

Cell type-specific TREs

In some embodiments, the E3-containing adenoviral vectors of the invention comprise a cell type-specific TRE, such as a prostate cell-specific TRE, a liver cell-specific TRE, a breast cancer cell-specific TRE, or a colon cancer cell-specific TRE. A cell type-specific TRE is preferentially functional in a specific type of cell relative to other types of cells of different functionality. For example, TREs that function preferentially in prostate cells (i.e., are prostate cell-specific) include, but are not limited to, TREs derived from the

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prostate-specific antigen gene (*PSA*-TRE) (U.S. Patent No. 5,648,478), the glandular
kallikrein-1 gene (from the human gene, *hKLK2*-TRE), and the probasin gene (*PB*-TRE)
(International Patent Application No. PCT/US98/04132). All three of these genes are
preferentially expressed in prostate cells and the expression is androgen-inducible.

5   Generally, expression of genes responsive to androgen induction requires the presence of
an androgen receptor (AR).

        In some embodiments, the E3-containing adenovirus vectors of the invention
comprise a PSA-TRE. PSA is synthesized exclusively by normal, hyperplastic, and
malignant prostatic epithelia; hence, its tissue-specific expression has made it an excellent

10   biomarker for benign prostatic hyperplasia (BPH) and prostatic carcinoma (CaP). Normal
serum levels of PSA are typically below 5 ng/ml, with elevated levels indicative of BPH or
CaP. Lundwall et al. (1987) *FEBS Lett.* 214: 317; Lundwall (1989) *Biochem. Biophys. Res.
Comm.* 161: 1151; and Riegmann et al. (1991) *Molec. Endocrin.* 5: 1921.

        The region of the *PSA* gene that is used to provide cell specificity dependent upon

15   androgens, particular in prostate cells, involves approximately 6.0 kilobases. Schuur et al.
(1996) *J. Biol. Chem.* 271:7043-7051. An enhancer region of approximately 1.5 kb in
humans is located between nt -5322 and nt -3739, relative to the transcription start site of
the *PSA* gene. The *PSA* promoter consists of the sequence from about nt -540 to nt +8
relative to the transcription start site. Juxtapositioning of these two genetic elements yield

20   a fully functional, minimal prostate-specific enhancer/promoter (*PSA*-TRE). Other
portions of the approximately 6.0 kb region of the *PSA* gene can be used in the present
invention, as long as requisite functionality is maintained. Accordingly, in some
embodiments, a *PSA*-TRE comprises nucleotides -5322 to -3739 juxtaposed to nucleotides
-540 to +8 relative to the transcription start site of the *PSA* gene.

25   In Example 1, adenoviral vector CN797 is described which comprises a composite
TRE comprising an HRE and a PSA-TRE, the PSA-TRE comprising a PSA enhancer from
-5322 to -3738 fused to a PSA promoter from -541 to +12. This PSA-TRE is derived from
adenoviral vector CN706. Rodriguez et al. (1997) *Cancer Research* 57:2559-2563.
Accordingly, in one embodiment an adenoviral vector comprises and adenovirus E1A gene

30   under transcriptional control of a composite TRE comprising the cell status-specific TRE,
HRE, and a cell type-specific TRE, a PSA-TRE.

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The *PSE* and *PSA* TRE used in the present invention are derived from sequences depicted in and Figure 22 (SEQ ID NO:9).

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a probasin TRE. The rat probasin (PB) gene encodes a nuclear and secreted
5   protein, probasin, that is only expressed in the dorsolateral prostate. Dodd et al. (1983) *J. Biol. Chem.* 258:10731–10737; Matusik et al. (1986) *Biochem. Cell. Biol.* 64: 601-607; and Sweetland et al. (1988) *Mol. Cell. Biochem.* 84: 3-15.

The PB-TREs used in this invention are derived from mammalian cells, including but not limited to, rodent cells, such as rat. In the Examples provided herein, a PB-TRE is
10   derived from rat cells. In one embodiment, the PB-TRE comprises a promoter of a probasin gene. In one embodiment, the PB-TRE comprises an enhancer from a probasin gene. In another embodiment, the PB-TRE comprises a promoter from a probasin gene and an enhancer from a probasin gene. In certain embodiments wherein the PB-TRE comprises an enhancer from a probasin gene, the enhancer may be in combination with a promoter
15   from a probasin gene or a promoter from another gene. In certain embodiments wherein the PB-TRE comprises a promoter from a probasin gene, the promoter may be in combination with an enhancer from a probasin gene or an enhancer from another gene. In addition, the PB-TRE can comprise multiple promoters and/or multiple enhancers derived from the probasin gene or another gene or other genes.

20   A DNA fragment comprising the 5'-flanking PB DNA, nt about -426 to about +28 (SEQ ID NO:13), carries sufficient information to direct prostate-specific, developmentally- and hormonally-regulated expression of a heterologous (non-probasin) gene in transgenic mice. Greenberg et al. (1994) *Mol. Endocrinol.* 8: 230-239; Foster et al. (1997) *Cancer Res.* 57:3325-30. Furthermore, this expression was both male-specific and
25   restricted to the epithelial cells of the lateral, dorsal, and ventral prostate lobes. The demonstration that the foreign gene activity approached precastration levels when transgenic mice were supplemented with testosterone indicates that the PB-driven reporter transgene was responding to androgens *in vivo*. Moreover, a PB-TRE could drive expression of the simian virus 40 large tumor antigen-coding region in the prostate of the
30   transgenic mice. Greenberg et al. (1995) *Proc. Natl. Acad. Sci.* 92: 3439-3443.

Accordingly, in one embodiment, a PB-TRE is the sequence upstream of the probasin coding segment, comprising, for example, the sequence given in SEQ ID NO:13. This sequence, e.g. from about -426 to about +28 relative to the transcriptional start site, comprises protein binding sites believed to be important or essential in cell-specific

5   transcription, including ARE-1, ARE-2, a CAAT box, and a TATAA box.

Alternatively, a PB-TRE comprises, for example, the fragment of DNA upstream of the PB gene between base pairs about -286 and about +28 relative to the transcriptional start (nucleotides about 141 to about 454 of SEQ ID NO:13). Rennie et al. (1993) *Mol. Endocrinol.* 7:23–36. Sequence analysis revealed that this PB-TRE contains two ARE sites

10   (designated ARE-1, also known as ARBS-1, which resembles a glucocorticoid response element, at about -236 to about -223 relative to the transcriptional start (nucleotides about 191 to about 204 of SEQ ID NO:13); and ARE-2, also known as ARBS-2, which is a unique sequence at about -140 to about -117 (nucleotides about 286 to about 310 of SEQ ID NO:13) required for androgen regulation. A single base mutation in ARE-1 or ARE-2

15   can result in the loss of androgen induction. Rennie et al. (1993) *Mol. Endocrinol.* 7:23-36. A fragment of 5'-flanking PB DNA containing the two ARE sites could drive expression of the bacterial chloramphenicol actyltransferase (CAT); expression was prostate-specific and inducible by androgens, but not by glucocorticoids. Greenberg et al. (1994) *Mol. Endocrinol.* 8:230–239. Like the probasin gene, the AR gene itself is regulated by two

20   ARE sites upstream of the coding segment. The first AR gene ARE site, ARE-1, resembles a half-site of the palindromic hormone response element and the second, ARE-2, is identical to a portion of the probasin sequence. Dai et al. (1996) *Mol. Endocrinol.* 10:1582-94. A PB enhancer is exemplified by an ARE site or pair of ARE sites, or any other sequence capable of assisting a promoter in prostate-specific transcription. Proper spacing

25   between ARE sites may also be important in their function.

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a human glandular kallikrein-2 TRE (*hKLK2*-TRE). The *hKLK2* gene encodes human glandular kallikrein-2 (hK2), a protein which is synthesized virtually exclusively in prostate cells and whose synthesis is upregulated by androgens, primarily by transcriptional

30   activation of *hKLK2*. Wolf et al. (1992) *Molec. Endocrinol.* 6:753-762; Morris (1989)

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Clin. Exp. Pharm. Physiol. 16:345-351; Qui et al. (1990) *J. Urol.* 144:1550-1556; Young et al. (1992) *Biochem.* 31:818-824.

hKLK2 promoter activity has been described . Schedlich et al. (1987) *DNA* 6:429-437; and Murtha et al. (1993) *Biochem.* 32:6459-6464. Promoter activity has been attributed to nucleotides about 11,290 to about 12,047 of SEQ ID NO:11. Accordingly, in some embodiments, an *hKLK2* TRE comprises an *hKLK2* promoter comprising nucleotides about 11,290 to about 12,047 of SEQ ID NO:11.

hKLK2 enhancer activity is found within nucleotides 1 through 9765 of SEQ ID NO:11 (corresponding to -12,014 to -2257 relative to the transcription start site). Portions of this region have been identified which retain enhancer function. Enhancer activity has been demonstrated in the region from nucleotides 8021 to 8371 of SEQ ID NO:11 (corresponding to -3993 to -3643 relative to the transcription start site), as described in PCT/US98/16312. Accordingly, the invention includes an isolated polynucleotide sequence comprising nucleotides about 8021 to about 8371 of SEQ ID NO:11. Enhancer activity has also been demonstrated in the region from nucleotides 7200 to 8371 of SEQ ID NO:11 (corresponding to -4814 to -3643 relative to the transcription start site), as described. Accordingly, the invention includes an isolated polynucleotide sequence comprising about 7200 to about 8371 of SEQ ID NO:11. Enhancer activity has further been demonstrated in the region from 6859 to 8627 of SEQ ID NO:11 (-5155 to -3387 relative to the transcription start site). Accordingly, the invention includes an isolated polynucleotide sequence comprising about 6859 to about 8627 of SEQ ID NO:11. Enhancer activity has been demonstrated in the region from 5976 to 9620 of SEQ ID NO:11 (-6038 to -2394 relative to the transcription start site). Accordingly, the invention includes an isolated polynucleotide sequence comprising about 5976 to about 9620 of SEQ ID NO:11. An active enhancer lies within an XhoI-ApaI fragment spanning a region from about 2 to about 6 kb upstream of the *hKLK2* structural gene. Accordingly, the invention further includes an isolated polynucleotide comprising nucleotides about 1 through about 9765 of SEQ ID NO:11. For each of these embodiments, the polynucleotide has enhancer activity.

In the present invention, cell type-specific TREs may be preferentially functional in particular tumor cells. Non-limiting examples of tumor cell-specific TREs, and non-

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limiting examples of respective potential target cells, include TREs from the following

genes: α-fetoprotein (*AFP*) (liver cancer), mucin-like glycoprotein DF3 (*MUC1*) (breast

carcinoma), carcinoembryonic antigen (*CEA*) (colorectal, gastric, pancreatic, breast, and

lung cancers), plasminogen activator urokinase (*uPA*) and its receptor gene (breast, colon,

5   and liver cancers), *HER-2/neu* (*c-erbB2/neu*) (breast, ovarian, stomach, and lung cancers).

Other cell type-specific TREs may be derived from the following exemplary genes

(cell type in which the TREs are specifically functional are in parentheses): vascular

endothelial growth factor receptor (endothelium), albumin (liver), factor VII (liver), fatty

acid synthase (liver), von Willebrand factor (brain endothelium), alpha-actin and myosin

10   heavy chain (both in smooth muscle), synthetase I (small intestine), Na-K-Cl transporter

(kidney). Additional cell type-specific TREs are known in the art, and some exemplary

TREs are discussed below.

In some embodiments, the E3-containing adenovirus vectors of the invention

comprise an AFP-TRE. AFP is an oncofetal protein, the expression of which is primarily

15   restricted to developing tissues of endodermal origin (yolk sac, fetal liver, and gut),

although the level of its expression varies greatly depending on the tissue and the

developmental stage. AFP is of clinical interest because the serum concentration of AFP is

elevated in a majority of hepatoma patients, with high levels of AFP found in patients with

advanced disease. The serum AFP levels in patients appear to be regulated by AFP

20   expression in hepatocellular carcinoma but not in surrounding normal liver. Thus, the AFP

gene appears to be regulated to hepatoma cell-specific expression.

Cell type-specific TREs from the *AFP* gene have been identified. For example, the

cloning and characterization of human AFP-specific enhancer activity is described in

Watanabe et al. (1987) *J. Biol. Chem.* 262:4812-4818. The entire 5' *AFP* flanking region

25   (containing the promoter, putative silencer, and enhancer elements) is contained within

approximately 5 kb upstream from the transcription start site.

The *AFP* enhancer region in human is located between about nt -3954 and about nt

-3335, relative to the transcription start site of the *AFP* gene. The human *AFP* promoter

encompasses a region from about nt -174 to about nt +29. Juxtapositioning of these two

30   genetic elements yields a fully functional *AFP*-TRE. This AFP-TRE is depicted in Figure

27. Ido et al. (1995) describe a 259 bp promoter fragment (nt -230 to nt +29) that is

specific for HCC. *Cancer Res.* 55:3105-3109. The *AFP* enhancer contains two regions, denoted A and B, located between nt -3954 and nt -3335 relative to the transcription start site. The promoter region contains typical TATA and CAAT boxes. Accordingly, in some embodiments, an AFP-TRE comprises nucleotides -3954 to -3335 relative to the AFP gene

5   transcription start site and a promoter. In other embodiments, an AFP-TRE comprises nucleotides -174 to +29 relative to the AFP gene transcription start site. In other embodiments, an AFP-TRE comprises nucleotides -3954 to -3335 juxtaposed to -174 to +29 relative to the AFP gene transcription start site.

Suitable target cells for adenoviral vectors containing *AFP*-TREs are any cell type

10   that allow an *AFP*-TRE to function. Preferred are cells that express, or produce, AFP, including, but not limited to, tumor cells expressing AFP. Examples of such cells are hepatocellular carcinoma cells, gonadal and other germ cell tumors (especially endodermal sinus tumors), brain tumor cells, ovarian tumor cells, acinar cell carcinoma of the pancreas (Kawamoto et al. (1992) *Hepatogastroenterology* 39:282–286), primary gall bladder tumor

15   (Katsuragi et al. (1989) *Rinsko Hoshasen* 34:371–374), uterine endometrial adenocarcinoma cells (Koyama et al. (1996) *Jpn. J. Cancer Res.* 87:612-617), and any metastases of the foregoing (which can occur in lung, adrenal gland, bone marrow, and/or spleen). In some cases, metastatic disease to the liver from certain pancreatic and stomach cancers produce AFP. Especially preferred are hepatocellular carcinoma cells and any of

20   their metastases. AFP production can be measured using assays standard in the art, such as RIA, ELISA or Western blots (immunoassays) to determine levels of AFP protein production or Northern blots to determine levels of AFP mRNA production. Alternatively, such cells can be identified and/or characterized by their ability to activate transcriptionally an *AFP*-TRE (i.e., allow an *AFP*-TRE to function).

25   In some embodiments, the E3-containing adenovirus vectors of the invention comprise a urokinase plasminogen activator TRE. The protein urokinase plasminogen activator (uPA) and its cell surface receptor, urokinase plasminogen activator receptor (uPAR), are expressed in many of the most frequently occurring neoplasia and appear to represent important proteins in cancer metastasis. Both proteins are implicated in breast,

30   colon, prostate, liver, renal, lung and ovarian cancer. Transcriptional regulatory elements that regulate uPA and uPAR transcription have been extensively studied. Riccio et al.

(1985) *Nucleic Acids Res.* 13:2759-2771; Cannio et al. (1991) *Nucleic Acids Res.* 19:2303–
2308.

In some embodiments, the E3-containing adenovirus vectors of the invention
comprise a carcinoembryonic antigen TRE. CEA is a 180,000-Dalton glycoprotein tumor-
associated antigen present on endodermally-derived neoplasia of the gastrointestinal tract,
such as colorectal, gastric (stomach) and pancreatic cancer, as well as other
adenocarcinomas such as breast and lung cancers. CEA is of clinical interest because
circulating CEA can be detected in the great majority of patients with CEA-positive
tumors. In lung cancer, about 50% of total cases have circulating CEA, with high
concentrations of CEA (greater than 20 ng/ml) often detected in adenocarcinomas.
Approximately 50% of patients with gastric carcinoma are serologically positive for CEA.

The 5' upstream flanking sequence of the *CEA* gene has been shown to confer cell-
specific activity. The *CEA* promoter region, approximately the first 424 nucleotides
upstream of the translational start site in the 5' flanking region of the gene, was shown to
confer cell-specific activity when the region provided higher promoter activity in CEA-
producing cells than in non-producing HeLa cells. Schrewe et al. (1990) *Mol. Cell. Biol.*
10:2738-2748. In addition, cell-specific enhancer regions have been found. WO 95/14100.
The entire 5' *CEA* flanking region (containing the promoter, putative silencer, and enhancer
elements) appears to be contained within approximately 14.5 kb upstream from the
translation start site. Richards et al. (1995); WO 95/14100. Further characterization of the
5' flanking region of the *CEA* gene by Richards et al. (1995) indicated two upstream
regions, -13.6 to -10.7 kb or -6.1 to -4.0 kb, when linked to the multimerized promoter
resulted in high-level and selective expression of a reporter construct in CEA-producing
LoVo and SW1463 cells. Richards et al. (1995) also localized the promoter region to nt -
90 and nt +69 relative to the translational start site, with region nt -41 to nt -18 as essential
for expression. WO95/14100 describes a series of 5' flanking *CEA* fragments which confer
cell-specific activity, such as about nt -299 to about nt +69; about nt -90 to about nt +69; nt
-14,500 to nt -10,600; nt -13,600 to nt -10,600, nt -6100 to nt -3800. In addition, cell
specific transcription activity is conferred on an operably linked gene by the *CEA* fragment
from nt -402 to nt +69, depicted in (SEQ ID NO:10). In the above-described CEA-TREs,
numbering is relative to the translation start site.

As shown in Example 4, cell type-specific transcription activity is also conferred on an operably linked gene by a CEA promoter fragment from nucleotide (nt) -300 to +70 relative to the CEA gene transcription start site (nucleotides 14161 to 14532 of the sequence depicted in Figure 23 (SEQ ID NO:10)). Accordingly, in some embodiments, an

5   E3-containing adenoviral vector of the invention comprises a CEA-TRE comprising a nucleotide sequence from about 14161 to about 14532 of SEQ ID NO:10), i.e., CN799, shown in Figure 16. This promoter fragment can be used alone or in combination with one or more enhancer fragments derived from a CEA gene 5' flanking region. Thus, as shown in Example 4, cell type-specific transcription activity is also conferred on an operably

10   linked gene by a CEA-TRE comprising a promoter fragment from -300 to +70 relative to the CEA gene transcription start site juxtaposed to a CEA enhancer fragment comprising nucleotides -6072 to -3815 relative to the CEA transcription start site (nucleotides 8392 to 10548 of the sequence depicted in Figure 23 (SEQ ID NO:10). Accordingly, in some embodiments, an E3-containing adenoviral vector of the invention comprises a CEA-TRE

15   comprising a nucleotide sequence from about 14161 to about 14532 of SEQ ID NO:10) operably linked to a nucleotide sequence from about 8392 to about 10548 of SEQ ID NO:10), i.e., CN798, shown in Figure 16. In other embodiments, a CEA-TRE comprises a CEA promoter fragment from about -300 to about +70 relative to the CEA transcription start site operably linked to a CEA enhancer fragment from about -6072 to about -3815 and

20   about -13.6 kb to about -10.7 kb relative to the CEA gene transcription start site. Any of the CEA-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Thus, any of the CEA-TREs may be used in the invention as long as requisite desired functionality is displayed in the adenovirus vector. The cloning and characterization of CEA sequences have been described in the literature and are thus

25   made available for practice of this invention and need not be described in detail herein.

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a mucin TRE. The protein product of the MUC1 gene (known as mucin or MUC1 protein; episialin; polymorphic epithelial mucin or PEM; EMA; DF3 antigen; NPGP; PAS-O; or CA15.3 antigen) is normally expressed mainly at the apical surface of

30   epithelial cells lining the glands or ducts of the stomach, pancreas, lungs, trachea, kidney, uterus, salivary glands, and mammary glands. Zotter et al. (1988) Cancer Rev. 11-12: 55-

101; and Girling et al. (1989) *Int. J. Cancer* 43: 1072–1076. However, mucin is
overexpressed in 75–90% of human breast carcinomas. Kufe et al. (1984) *Hybridoma* 3:
223–232. For reviews, see Hilkens (1988) *Cancer Rev.* 11–12: 25–54; and Taylor-
Papadimitriou, et al. (1990) *J. Nucl. Med. Allied Sci.* 34: 144–150. Mucin protein
5   expression correlates with the degree of breast tumor differentiation. Lundy et al. (1985)
Breast Cancer Res. Treat. 5: 269–276. This overexpression appears to be controlled at the
transcriptional level.

Overexpression of the *MUC1* gene in human breast carcinoma cells MCF-7 and
ZR-75-1 appears to be regulated at the transcriptional level. Kufe et al. (1984); Kovarik
10   (1993) *J. Biol. Chem.* 268:9917–9926; and Abe et al. (1990) *J. Cell. Physiol.* 143: 226–231.
The regulatory sequences of the *MUC1* gene have been cloned, including the
approximately 0.9 kb upstream of the transcription start site which contains a TRE that
appears to be involved in cell-specific transcription. Abe et al. (1993) *Proc. Natl. Acad.
Sci. USA* 90: 282-286; Kovarik et al. (1993); and Kovarik et al. (1996) *J. Biol. Chem.*
15   271:18140–18147.

Any *MUC1*-TREs used in the present invention are derived from mammalian cells,
including but not limited to, human cells. Preferably, the *MUC1*-TRE is human. In one
embodiment, the *MUC1*-TRE may contain the entire 0.9 kb 5' flanking sequence of the
MUC1 gene. In other embodiments, the *MUC1*-TREs comprise the following sequences
20   (relative to the transcription start site of the *MUC1* gene): about nt -725 to about nt +31, nt
-743 to about nt +33, nt -750 to about nt +33, and nt -598 to about nt +485 (operably-linked
to a promoter).

In some embodiments, the E3-containing adenovirus vectors of the invention
comprise a c-erbB2/neu TRE. The *c-erbB2/neu* gene (*HER-2/neu* or *HER*) is a
25   transforming gene that encodes a 185 kD epidermal growth factor receptor-related
transmembrane glycoprotein. In humans, the c-erbB2/neu protein is expressed during fetal
development, however, in adults, the protein is weakly detectable (by
immunohistochemistry) in the epithelium of many normal tissues. Amplification and/or
over-expression of the *c-erbB2/neu* gene has been associated with many human cancers,
30   including breast, ovarian, uterine, prostate, stomach and lung cancers. The clinical
consequences of the c-erbB2/neu protein over-expression have been best studied in breast

and ovarian cancer. c-erbB2/neu protein over-expression occurs in 20 to 40% of
intraductal carcinomas of the breast and 30% of ovarian cancers, and is associated with a
poor prognosis in subcategories of both diseases. Human, rat and mouse *c-erbB2/neu*
TREs have been identified and shown to confer *c-erbB2/neu* expressing cell specific

5   activity. Tal et al. (1987) *Mol. Cell. Biol.* 7:2597–2601; Hudson et al. (1990) *J. Biol.*
Chem. 265:4389–4393; Grooteclaes et al. (1994) *Cancer Res.* 54:4193–4199; Ishii et al.
(1987) *Proc. Natl. Acad. Sci. USA* 84:4374–4378; Scott et al. (1994) *J. Biol. Chem.*
269:19848–19858.

The cell type-specific TREs listed above are provided as non-limiting examples of

10   TREs that would function in the instant invention. Additional cell type-specific TREs are
known in the art, as are methods to identify and test cell specificity of suspected cell type-
specific TREs.

Cell status-specific TREs

Cell status-specific TREs for use in the E3-containing adenoviral vectors of the

15   present invention can be derived from any species, preferably a mammal. A number of
genes have been described which are expressed in response to, or in association with, a cell
status. Any of these cell status-associated genes may be used to generate a cell status-
specific TRE.

An example of a cell status is cell cycle. An exemplary gene whose expression is

20   associated with cell cycle is E2F-1, a ubiquitously expressed, growth-regulated gene, which
exhibits peak transcriptional activity in S phase. Johnson et al. (1994) *Genes Dev.* 8:1514-
1525. The RB protein, as well as other members of the RB family, form specific
complexes with E2F-1, thereby inhibiting its ability to activate transcription. Thus, E2F-1-
responsive promoters are down-regulated by RB. Many tumor cells have disrupted RB

25   function, which can lead to de-repression of E2F-1-responsive promoters, and, in turn, de-
regulated cell division.

Accordingly, in one embodiment, the invention provides an E3-containing
adenoviral vector in which an adenoviral gene (preferably a gene necessary for replication)
is under transcriptional control of a cell status-specific TRE, wherein the cell status-specific

30   TRE comprises a cell cycle-activated TRE. In one embodiment, the cell cycle-activated

TRE is an E2F1 TRE. In one embodiment, this TRE comprises the sequence depicted in Figure 21 (SEQ ID NO:8).

Another group of genes that are regulated by cell status are those whose expression is increased in response to hypoxic conditions. Bunn and Poyton (1996) *Physiol. Rev.*
5   76:839-885; Dachs and Stratford (1996) *Br. J. Cancer* 74:5126-5132; Guillemin and Krasnow (1997) *Cell* 89:9-12. Many tumors have insufficient blood supply, due in part to the fact that tumor cells typically grow faster than the endothelial cells that make up the blood vessels, resulting in areas of hypoxia in the tumor. Folkman (1989) *J. Natl. Cancer Inst.* 82:4-6; and Kallinowski (1996) *The Cancer J.* 9:37-40. An important mediator of
10   hypoxic responses is the transcriptional complex HIF-1, or hypoxia inducible factor-1, which interacts with a hypoxia-responsive element (HRE) in the regulatory regions of several genes, including vascular endothelial growth factor, and several genes encoding glycolytic enzymes, including enolase-1. Murine HRE sequences have been identified and characterized. Firth et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500. An HRE from
15   a rat enolase-1 promoter is described in Jiang et al. (1997) *Cancer Res.* 57:5328-5335. An HRE from a rat enolase-1 promoter is depicted in Figure 20 (SEQ ID NO:7).

Accordingly, in one embodiment, an E3-containing adenovirus vector comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a cell status-specific TRE comprising an HRE. In one
20   embodiment, the cell status-specific TRE comprises the HRE depicted in Figure 20 (SEQ ID NO:7).

Other cell status-specific TREs include heat-inducible (i.e., heat shock) promoters, and promoters responsive to radiation exposure, including ionizing radiation and UV radiation. For example, the promoter region of the early growth response-1 (Egr-1) gene
25   contains an element(s) inducible by ionizing radiation. Hallahan et al. (1995) *Nat. Med.* 1:786-791; and Tsai-Morris et al. (1988) *Nucl. Acids. Res.* 16:8835-8846. Heat-inducible promoters, including heat-inducible elements, have been described. See, for example Welsh (1990) in "Stress Proteins in Biology and Medicine", Morimoto, Tisseres, and Georgopoulos, eds. Cold Spring Harbor Laboratory Press; and Perisic et al. (1989) *Cell*
30   59:797-806. Accordingly, in some embodiments, the cell status-specific TRE comprises an element(s) responsive to ionizing radiation. In one embodiment, this TRE comprises a 5'

flanking sequence of an Egr-1 gene. In other embodiments, the cell status-specific TRE comprises a heat shock responsive element.

The cell status-specific TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell status-specific TREs are

5   known in the art, as are methods to identify and test cell status specificity of suspected cell status-specific TREs.

Adenovirus genes under control of a target cell-specific TRE

In the E3-containing adenoviral vectors of the present invention, an adenovirus gene, preferably an adenovirus gene essential for replication, is under control of a target

10   cell-specific TRE.

Preferably, the gene(s) is an early gene, such as E1A, E1B, E2, and/or E4. Embodiments in which E3 is under transcriptional control of a target cell-specific TRE have been discussed above. More preferably, the early gene(s) under target cell-specific TRE control is E1A and/or E1B. More than one early gene can be placed under control of

15   an target cell-specific TRE. Example 1 provides a more detailed description of such constructs.

The E1A gene is expressed immediately after viral infection (0-2 hours) and before any other viral genes. E1A protein acts as a _trans_-acting positive-acting transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2,

20   E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early times after Ad5 infection. Flint (1982) _Biochem. Biophys. Acta_ 651:175-208; Flint (1986) _Advances Virus Research_ 31:169-228; Grand (1987) _Biochem. J._ 241:25-38. In the absence of a functional E1A gene, viral infection does not proceed, because the gene

25   products necessary for viral DNA replication are not produced. Nevins (1989) _Adv. Virus Res._ 31:35-81. The transcription start site of Ad5 E1A is at nucleotide 498 and the ATG start site of the E1A protein is at nucleotide 560 in the virus genome.

The E1B protein functions _in trans_ and is necessary for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of

30   late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been implicated as the defining element of difference in the host range of Ad40

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and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis. Bailey, Mackay et al. (1993) *Virology* 193:631; Bailey et al. (1994) *Virology* 202:695-706). The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box.

5   The E2 region of adenovirus codes for proteins related to replication of the adenoviral genome, including the 72 kDa DNA-binding protein, the 80 kD precursor terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during

10   late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable to genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33 kD

15   protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position nt 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor biding sites E2F and ATF. Therefore, insertion of a target cell-specific TRE having SpeI ends into the SpeI site in the +-strand would disrupt the

20   endogenous E2 early promoter of Ad5 and should allow target cell-restricted expression of E2 transcripts.

The E4 gene has a number of transcription products. The E4 region codes for two polypeptides which are responsible for stimulating the replication of viral genomic DNA and for stimulating late gene expression. The protein products of open reading frames

25   (ORFS) 3 and 6 can both perform these functions by binding the 55kD protein from E1B and heterodimers of E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55kD protein for activity while the ORF 3 protein does not. In the absence of functional protein from ORF 3 and ORF 6, plaques are produced with an efficiency less than 10^{-6} that of wild type virus. To further restrict viral replication to cells exhibiting a requisite

30   physiological condition or state, E4 ORFs 1-3 can be deleted, making viral DNA replication and late gene synthesis dependent on E4 ORF 6 protein. By combining such a

mutant with sequences in which the E1B region is regulated by a target cell-specific TRE, a virus can be obtained in which both the E1B function and E4 function are dependent on a target cell-specific TRE driving E1B.

The major late genes relevant to the subject invention are genes L1, L2, L3, L4, and L5 which encode proteins of the adenovirus virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. The late genes are all under the control of the major late promoter (MLP), which is located in Ad5 at nucleotides +5986 to +6048.

Transgenes

In addition to conferring selective cytotoxic and/or cytolytic activity by virtue of preferential replication competence in target cells, the adenovirus vectors of this invention can further include a heterologous gene (transgene) under the control of a heterologous TRE. In this way, various genetic capabilities may be introduced into target cells, particularly cancer cells. For example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the cancerous target cell. This could be accomplished by coupling the target cell-specific cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or abrin (Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7: 1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992) *Neuron* 8: 507; Piatak et al. (1988) *J. Biol. Chem.* 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415), genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly; genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. awsin, papain, proteinase K,

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carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN-α, -β, -γ, TNF-α, -β, TGF-α, -β, NGF, and the like. The positive effector genes could be used in an earlier phase, followed by cytotoxic activity due to replication.

Preparation of the adenoviral vectors of the invention

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Any of the various serotypes of adenovirus can be used in the present invention, such as Ad2, Ad5, Ad12 and Ad40. For purposes of illustration, serotype Ad5 is exemplified herein.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art, such as chemical synthesis, by recombinant methods, and/or by obtaining the desired sequence(s) from biological sources.

Adenoviral vectors are conveniently prepared by employing two plasmids, one plasmid providing for the left hand region of adenovirus and the other plasmid providing for the right hand region, where the two plasmids share at least about 500 nucleotides of middle region for homologous recombination. In this way, each plasmid, as desired, may be independently manipulated, followed by cotransfection in a competent host, providing complementing genes as appropriate. Plasmids are generally introduced into a suitable host cell such as 293 cells using appropriate means of transduction, such as lipid-containing agents (e.g., cationic liposomes). Alternatively, *in vitro* ligation of the right and left-hand portions of the adenovirus genome can also be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner et al. (1983) *Nucleic Acids Research* 11: 6003-6020; Bridge et al. (1989) *J. Virol.* 63: 631-638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5. pBHGE3 (Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, including E3.

For manipulation of the early genes, the transcription start site of Ad5 E1A is at nt 498 and the ATG start site of the E1A protein is at nt 560 in the virus genome. This region

can be used for insertion of a target cell-specific TRE. A restriction site may be introduced

by employing polymerase chain reaction (PCR), where the primer that is employed may be

limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5

genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in

5   the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two

steps, where overlapping primers at the center of the region introduce a nucleotide

sequence change resulting in a unique restriction site, one can provide for insertion of

target cell-specific TRE at that site. Example 1 provides a more detailed description of an

adenoviral vector in which E1A is under target cell-specific TRE control.

10   A similar strategy may also be used for insertion of a heterologous TRE to regulate

E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl

and a TATA box. This region extends from nt 1636 to nt 1701. By insertion of a

heterologous TRE in this region, one can provide for target cell-specific transcription of the

E1B gene. By employing the left-hand region modified with a heterologous TRE

15   regulating E1A as the template for introducing a heterologous TRE to regulate E1B, the

resulting adenovirus vector will be dependent upon the target cell-specific transcription

factors for expression of both E1A and E1B.

            Similarly, a heterologous TRE can be inserted upstream of the E2 gene to make its

expression target cell specific. The E2 early promoter, mapping in Ad5 from nt 27050-

20   27150, consists of a major and a minor transcription initiation site, the latter accounting for

about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription

factor binding sites and an ATF transcription factor binding site. For a detailed review of

the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Microbiol. and

Immunol.* (1995) 199 (part 3):177-194.

25   For E4, one must use the right hand portion of the adenovirus genome. The E4

transcription start site is predominantly at 35609, the TATA box at nt 35638 and the first

ATG/CTG of ORF 1 is at nt 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any

of the above strategies for the other genes, a heterologous TRE may be introduced

upstream from the transcription start site. For the construction of mutants in the E4 region,

30   the co-transfection and homologous recombination are performed in W162 cells (Weinberg

et al. (1983) *Proc. Natl. Acad. Sci. USA* 80:5383–5386) which provide E4 proteins *in trans*

to complement defects in synthesis of these proteins. Alternatively, these constructs can be produced by *in vitro* ligation.

Adenoviral constructs containing an E3 sequence can be generated as described in Example 1, wherein homologous recombination between an E3-containing adenoviral plasmid, for example, BHGE3 (Microbix Biosystems Inc., Toronto) and a non-E3-containing adenoviral plasmid, is carried out.

Alternatively, an adenoviral vector comprising an E3 sequence can be introduced into cells, for example 293 cells, along with an adenoviral construct or an adenoviral plasmid construct, where they can undergo homologous recombination to yield adenovirus containing an E3 region (or portion of E3). In this case, the E3-containing adenoviral vector and the adenoviral construct or plasmid construct contain complementary regions of adenovirus, for example, one contains the left-hand and the other contains the right-hand region, with sufficient sequence overlap as to allow homologous recombination.

Alternatively, an E3-containing adenoviral vector of the invention can be constructed using other conventional methods including standard recombinant methods (e.g., using restriction nucleases and/or PCR), chemical synthesis, or a combination of any of these. Further, deletions of portions of the E3 region can be created using standard techniques of molecular biology.

Host cells comprising the adenoviral vectors of the invention

The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequences requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Prokaryotic host cells include bacterial·cells, for example, *E. coli* and mycobacteria. Among eukaryotic host cells are yeast, insect, avian, plant and mammalian cells. Host systems are known in the art and need not be described in detail herein.

Compositions of the invention

The present invention also provides compositions, including pharmaceutical compositions, containing an adenoviral vector(s) described herein. Such compositions (especially pharmaceutical compositions) are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an

individual. Pharmaceutical compositions, comprised an adenoviral vector of this invention in a pharmaceutically acceptable excipient (generally an effective amount of the adenoviral vector), are suitable for systemic or localized administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral

5   solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing (1990). Pharmaceutical compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the

10   invention.

Other compositions are used, and are useful for, detection methods described herein. For these compositions, the adenoviral vector usually is suspended in an appropriate solvent or solution, such as a buffer system. Such solvent systems are well known in the art.

15   *Kits of the invention*

The present invention also encompasses kits containing an adenoviral vector(s) of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits

20   embodied by this invention allow detection of the presence of cells which allow a target cell-specific TRE to function, including, for example, neoplastic cells, in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the

25   procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Methods using the adenovirus vectors of the invention

The adenoviral vectors of the invention can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention

30   includes methods using the adenoviral vectors described above.

51

In one embodiment, methods are provided for conferring selective cytotoxicity in target cells (i.e., cells which allow a target cell-specific TRE to function) comprising contacting the cells with an adenovirus vector described herein, such that the adenovirus vector enters the cell (and subsequently replicates). Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ^{3}H-thymidine incorporation, and/or lysis.

In another embodiment, methods are provided for propagating an adenovirus specific for mammalian cells which allow a target cell-specific TRE to function. These methods entail combining an adenovirus vector with mammalian cells, whereby said adenovirus enters the cell and is propagated.

The invention further provides methods of suppressing tumor cell growth comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell (and/or whereby introduction of the adenoviral vector results in suppression of tumor growth). Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells.

The invention also includes methods for detecting target cells in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. For these methods, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. A suitable biological sample is one in which target cells, for example, cancerous cells, may be or are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which cancerous cells exhibiting a requisite physiological state, such as cells within a solid tumor which are under hypoxic conditions, or cancerous or non-cancerous cells of a particular cell type, are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay conditions, such as selective enrichment, and/or solubilization. In these methods, target cells can be detected using *in vitro* assays that detect adenoviral proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yield) and plaque assays (which measure infectious particles

per cell). Propagation can also be detected by measuring specific adenoviral DNA replication, which are also standard assays.

The adenoviral vectors of the invention can be used alone or in conjunction with other active agents, such as chemotherapeutics, that promote the desired objective.

5   The adenoviral vectors can be used in a variety of forms, including, but not limited to, naked polynucleotide (usually DNA) constructs; polynucleotide constructs complexed with agents to facilitate entry into cells, such as cationic liposomes or other cationic compounds such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more immunogenic); packaged into other particulate viral

10   forms such as HSV or AAV; complexed with agents (such as PEG) to enhance or dampen an immune response; complexed with agents that facilitate *in vivo* transfection, such as DOTMA™, DOTAP™, and polyamines.

If an adenoviral vector is packaged into an adenovirus, the adenovirus itself may also be selected to further enhance targeting. For example, adenovirus fibers mediate

15   primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Virol.* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type and/or reduced affinity for non-target cell types, such subgenus (or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

20   The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art (such as calcium phosphate precipitation or electroporation), direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells

25   (i.e., whether the cells are *in vitro* or *in.vivo*).

If used as a packaged adenovirus, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to about 10^{14}. The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 µg to

30   about 1000 µg of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, whether systemically or locally, depending upon the

intended use and the immune response potential of the host, and may also be administered

as multiple, simultaneous injections. If an immune response is undesirable, the immune

response may be diminished by employing a variety of immunosuppressants, so as to

permit repetitive administration, without a strong immune response. If packaged as another

5   viral form, such as HSV, an amount to be administered is based on standard knowledge

about that particular virus (which is readily obtainable from, for example, published

literature) and can be determined empirically.

The following examples are provided to illustrate but not limit the invention.

10

EXAMPLES

EXAMPLE 1

Construction of target cell-specific adenoviral constructs containing an E3 region

Target cell-specific adenoviral vectors were constructed by first generating target

15   cell-specific adenoviral vectors and/or adenoviral plasmid vectors lacking an E3 region,

then recombining these vectors with E3-containing adenoviral constructs.

With respect to adenoviral constructs (as opposed to precursor plasmid constructs),

it is understood that "CN" and "CV" designations may be used interchangeably. For

example, CN787 and CV787 refer to the same adenoviral construct.

20   *Generation of adenoviruses and adenoviral plasmid vectors that contain target cell-specific*
TREs driving expression of E1A and/or E1B

A human embryonic kidney cell line, 293, efficiently expresses E1A and E1B genes

of Ad5 and exhibits a high transfection efficiency with adenovirus DNA. For these

experiments, 293 cells were co-transfected with one left end Ad5 plasmid and one right end

25   Ad5 plasmid. Homologous recombination generates adenoviruses with the required

genetic elements for replication in 293 cells which provide E1A and E1B proteins *in trans*

to complement defects in synthesis of these proteins.

The plasmids to be combined were co-transfected into 293 cells using cationic

liposomes such as Lipofectin (DOTMA:DOPE™, Life Technologies) by combining the

30   two plasmids, then mixing the plasmid DNA solution (10 μg of each plasmid in 500 μl of

minimum essential medium (MEM) without serum or other additives) with a four-fold

molar excess of liposomes in 200 μl of the same buffer. The DNA-lipid complexes were then placed on the cells and incubated at 37°C, 5% CO_2 for 16 hours. After incubation the medium was changed to MEM with 10% fetal bovine serum and the cells are further incubated at 37°C, 5% CO_2, for 10 days with two changes of medium. At the end of this

5   time the cells and medium were transferred to tubes, freeze-thawed three times, and the lysate was used to infect 293 cells at the proper dilution to detect individual viruses as plaques.

Plaques obtained were plaque purified twice, and viruses were characterized for presence of desired sequences by PCR and occasionally by DNA sequencing. For further

10   experimentation, the viruses were purified on a large scale by cesium chloride gradient centrifugation.

CN739

An adenovirus vector in which expression of the E1A gene is under control of the *PB*-TRE and expression of the E1B gene is under control of the prostate specific antigen

15   transcriptional regulatory element (*PSA*-TRE) was constructed as follows. The *PSA*-TRE region has been described in detail in, inter alia, U.S. Patent Nos. 5,648,478 and 5,698,443; Lundwall (1989) *Biochim. Biophys. Res. Commun.* 161:1151-1159; and Zhang et al. (1997) *Nucleic Acids Res.* 25:3143-50.

The PinAI *PB*-TRE fragment was inserted into CN125 digested with PinAI, which

20   cleaves just upstream of E1A, to create construct CN257, which is a plasmid containing a *PB*-TRE (from -426 to +28 relative to the rat probasin gene transcription start site) operably linked to the E1A gene and a *PSA*-TRE (from -5322 to -3738 and -541 to +12 relative to the human *PSA* gene transcription start site) operably linked to the E1B gene.

Adenoviruses that contain two heterologous TREs were generated by homologous

25   recombination in 293 cells. Briefly, 5 μg of CN257 and 5 μg of BHG10, which contains the right hand end of Ad5, was co-transfected into 293 cells. The cells were overlaid with medium, and infectious virus, generated by *in vivo* recombination, was detected by cytopathic effect and isolated. Plaque-purified stocks of an adenovirus vector, designated CN739, were established. The structure of the recombinant virus was characterized by

30   PCR, restriction endonuclease digestion and Southern blot. The viral genome of CN739

has the E1A transcription unit of Ad5 under the control of *PB*-TRE while E1B is under the control of *PSA*-TRE.

<u>CN732</u>

CN732 (also referred to as CV732) which contains AFP-TRE driving expression of the E1A gene, was constructed by PCR as described below. CN732 contains the AFP enhancer domain (-3954 to -3335) and the promoter (-174 to +29) cloned into an engineered AgeI site at nt 547 of Ad5 to drive Ad5 E1a expression.

pXC.1 contains the left 16 mu of Ad5. pBHGE3 from Microbix, Inc., Toronto, Canada contains the circularized Ad5 genome with an E1 deletion extending to the packaging signal. Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806; and McKinnon et al. (1982) *Gene* 19:33-42. A platform plasmid (CP124) with two unique restriction sites was constructed from pXC.1. CP124 has an AgeI site at bp 547 between the E1a mRNA cap site and E1a translation initiation site, and an EagI site at bp 1681 between the E1b promoter and E1b mRNA cap site. To construct viruses that preferentially replicate in AFP+ cells, a composite AFP TRE of the enhancer and promoter was assembled by overlap PCR and cloned into CP124. The enhancer element (-3856 to -3267) was amplified from human genomic DNA (Clontech, Palo Alto, CA) with primers

5' GTGACCGGTG CATTGCTGTG AACTCTGTA 3' (39.055B) (SEQ ID NO:); and

5' ATAAGTGGCC TGGATAAAGC TGAGTGG 3' (38.055D) (SEQ ID NO:).

The promoter (-163 to +34) was amplified with primers

5' GTCACCGGTC TTTGTTATTG GCAGTGGT 3' (39.055J) (SEQ ID NO:); and

5' ATCCAGGCCA CTTATGAGCT CTGTGTCCTT 3' (39.055M) (SEQ ID NO:).

Underlined regions in primers 39.055B and 39.055J indicate AgeI sites. The two products were annealed and used as the template for PCR with primers 39.055B and 39.055J. This overlap product was digested with PinAI (an AgeI isoschizomer) and ligated to PinAI cut CP124.

The resulting plasmid, CN219, contains the AFP TRE driving the E1A gene. CN732 was generated via homologous recombination by cotransfecting 293 cells with CN219 with pBHG10. pBHG10 is a plasmid that contains all of the Ad5 sequences except the E1 region as well as a deletion in the E3 region from Ad5 bp 28133 to 30818.

<u>CN733</u>

CN733 (also referred to as CV733) which contains two AFP-TREs driving

expression of the E1A and E1B genes, was constructed by using the following two PCR

primers to amplify the enhancer/promoter element described above (-3954 to -3335 and -

5   174 to +29):

5' TATCGGCCGG CATTGCTGTG AACTCT 3' (39.077A) (SEQ ID NO:1); and

5' TTACGGCCGC TTTGTTATTG GCAGTG 3' (39.077C) (SEQ ID NO:2).

The PCR product was digested with EagI and ligated into similarly cut CN219. The

resulting plasmid, CN224, contains two identical AFP regulatory elements, one each

10   modulating expression of the E1A gene and the E1B gene. CN733 was generated by

homologous recombination in 293 cells by cotransfecting CN224 and BHG10.

<u>CN796</u>

Adenoviral vector CN796 comprises a composite TRE comprising an HRE and a

PSA-TRE, the PSA-TRE comprising a PSA enhancer from -5322 to -3738 fused to a PSA

15   promoter from -541 to +12. This PSA-TRE is derived from adenoviral vector CN706.

Rodriguez et al. (1997) *Cancer Research* 57:2559-2563.

CN796 was made by co-transfecting CN515 with pBHG10. CN515 was

constructed by inserting a 67 base pair fragment from HRE eno1 (Jiang et al. (1997)

Cancer Research 57:5328-5335) (SEQ ID NO:7; Figure 20) into CN65 at the BglII site.

20   CN65 is a plasmid containing an enhancer and promoter from the human PSA gene,

consisting of an enhancer from -5322 to -3738 fused to a PSA promoter from -541 to +12.

This is the PSA-TRE contained within plasmid CN706.

<u>CN532</u>

CEA-TRE-containing adenoviral vector CN532 was generated as follows. The

25   native E1A promoter was deleted (as a 64 bp fragment) from CN124 (wild-type left-hand

end of Ad5) to generate CN306. CN143 is a pBluescript (Stratagene, La Jolla, California)

derivative containing a PSA-TRE fragment. The PSA-TRE fragment from CN143 was

then inserted upstream of the promoter-less E1A coding segment of CN306 to generate

CN321.

CN532 was constructed from CN321 by replacing the *PSA*-TRE of CN321 with a CEA promoter. The CEA promoter was amplified from human genomic DNA by PCR using two primers:

5' GATCTCGAGA CCCGGGACCC TGCTGGGTTT C 3' (76.13.1) (SEQ ID NO:3); and

5' GATCACCGGT GCTTGAGTTC CAGGAACGTT TTG 3' (76.13.2) (SEQ ID NO:4).

The PCR product was enzymatically digested with PinAI, then ligated to PinAI-cut CN321 vector portion. The CEA promoter fragment amplified by these primers corresponds to -300 to +70 relative to the CEA gene transcription start site.

CN525

As with CN532, CN525 was generated from CN321 by replacing the PSA-TRE with a CEA promoter and enhancer. The CEA enhancer was amplified from human genomic DNA by PCR using two synthetic oligonucleotide primers:

5' GATCAACCGG TACCGACTTC TGTAGCTTTG GGAAGG 3' (76.13.3) (SEQ ID NO:5); and

5' GATCCTCGAG CCCGGGTTCA AGCAATTCTC CTGC 3' (76.13.4) (SEQ ID NO:6).

The PCR product was cut with PinAI, ligated to the CEA promoter fragment described above, and ligated to PinAI-cut CN321. The orientation of the enhancer fragment is 3' to 5' relative to the promoter. The CEA enhancer fragment amplified by 76.13.3 and 76.13.4 corresponds to -6072 to -3815 relative to the CEA gene transcription start site. Thus, CN525 contains a CEA-TRE comprising a CEA enhancer from -6072 to -3815 juxtaposed to a CEA promoter from -300 to +70 relative to the CEA gene transcription start site.

Generation of recombinant adenoviruses containing E3

Adenovirus containing E3 were generated by homologous recombination in 293 cells. Briefly, CN257 was co-transfected with pBHGE3 (Microbix) into 293 cells. The cells were overlaid with media, and infectious virus generated by *in vivo* recombination was detected by cytopathic effect and isolated. Plaque-purified stocks of an adenovirus vector, designated CN787, were established. The structure of the recombinant virus was characterized by PCR, restriction endonuclease digestion and Southern blot. The resulting

recombinant virus, CN787 (also referred to as CV787), is a full-length Ad5 with a *PB*-TRE driving the expression of E1A and a *PSA*-TRE driving expression of E1B, and which also contains an E3 gene. Thus, CN787 is analogous to CN739, except that it contains an E3 region. This construct is depicted schematically in Figure 2.

5   Using this method, CN790, CN797, CN798, CN799, and CN802 (also referred to as CV790, CV797, CV798, CV799 and CV802, respectively) were generated by recombination between BHGE3 and CN733, CN796, CN532, CN525, and pXC.1, respectively. Plasmid pXC.1 (Microbix) is wild-type adenovirus Type 5. CN790 is the same as CN733 but with the addition of the Ad5 full-length E3 region. CN790 is depicted

10   schematically in Figure 11; CN798, CN799, and CN802 are depicted schematically in Figure 16; and CN797 is depicted schematically in Figure 19.

Structure, Genomic Stability, and Self-Inactivation of Viruses

15   Virus genetic structures were confirmed by PCR and Southern blot analysis.

Southern Blot Analysis. 10 ng of viral DNA (QIAmp blood kit, Qiagen) was digested with AflII or AflII and ClaI. DNA was fractionated through a 1% agarose gel and transferred by capillary transfer to a Nytran nylon membrane (Schleicher and Schuell). Viral DNA was probed with $[\alpha\text{-}^{32}P]$ dCTP labeled PCR products specific for E1a or E1b

20   sequences. The E1A probe was made by PCR from CN706 DNA amplifying a 938bp fragment of Ad5 DNA with primers

5' GAGACATATT ATCTGCCACG G 3' (18.184.1) (SEQ ID NO:); and

5' CGTTAAGCAA GTCCTCGATA C 3' (18.184.2) (SEQ ID NO:),

and the E1b probe was made from CN706 DNA using primers

25   (5' TTGGTTTTGG AGGTTTCTGT GGGG 3' (46.135B) (SEQ ID NO:); and

(5' AAAGGCCACC CTATCCTCCG TATC 3' (46.135E) (SEQ ID NO:) (881bp product).

Blots were hybridized overnight at 45°C in ZipHyb solution (Ambion), washed two times in 2XSSC, 0.1%SDS at room temperature and two times in 0.1XSSC, 0.1 %SDS at 65°C. Blots were visualized by exposure in a GS-525 Molecular Imager (BioRad Laboratories).

30   The proportion of CN733 or CN790 deletion mutant (ΔE1a CN733 or ΔE1a CN790) in each stock was estimated by determining the amount of radioactivity in bands

corresponding to the CN733 or CN790 (5.2 kb band, AflII digest and E1b probe) and the

ΔE1a CN733 or ΔE1a CN790 (3.2 kb band) using the Molecular Imager and Multi-Analyst

imaging software (Bio-Rad, version 1.0.2).

     Results. Viral DNA was digested with AflII to release the El region and probed

5   with [α-^{32}P] dCTP labeled DNA specific for the E1a gene (Figure 29A) and the E1b gene

(Figure 29B). The expected fragment sizes are shown in Table 1.

10

Table 1

Expected Fragment Sizes (kb)

Virus	E1A Probe AflII	E1B Probe AflII
CN702	3.5	3.5
CN732	4.4	4.4
CN733	5.2	5.2
ΔE1a CN733	-	3.2
CN734	4.4·	4.4
CN739	5.7	5.7
CN790	5.2	5.2
ΔE1a CN790	-	3.2

All the viruses had the expected structure and CN702, CN732, CN734 and CN739 were

stable through three passages. However, preparations of CN733 and CN790 showed the

15   expected virus, indicated by a 5.2 kb band, but also contained an additional 3.2kb band

(Figure 29B). This band did not appear in Southern blots probed with E1a but did appear

in Southern blots probed with E1b. The DNA of individual clones of CN733 and CN790

were cloned and their DNAs sequenced. DNA sequence analysis of individual clones

indicated homologous recombination occurred between the two AFPs and the AFP DNA

20   segments during propagation, resulting in the deletion of the E1a gene and one copy of the

AFP TRE. These deletion viruses were named ΔE1a CN733 and ΔE1a CN790

respectively. The rate of accumulation of ΔE1a CN733 and ΔE1a CN790 in stocks of

CN733 and CN790 was evaluated by performing Southern blot analysis on several viral

stocks prepared in series. Data presented in Figure 30 indicate that ΔE1a CN790 was 7% at

the first passage and accumulated to about 14% of the total viral particles by the third

passage in 293 cells. ΔE1a CN790 accumulated rapidly in cells that did not select against

the replication defective mutant like 293 cells or in cells that had been infected at an MOI =

10. However, CN790 prepared at low MOI = 0.1 in AFP⁺ HepG2 cells that did not support

5   replication defective adenovirus replication was devoid of the deletion mutant after three

passages (Figure 30).

EXAMPLE 2

*In vitro and in vivo characterization of CN787, an E3-containing adenoviral construct
comprising a PB-TRE driving expression of E1A and a PSA-TRE driving expression of*
10   *E1B*

Cells and Culture Methods

LNCaP cells were obtained at passage 9 from the American Type Culture

Collection (Rockville, MD). LNCaP cells were maintained in RPMI 1640 medium (RPMI)

15   supplemented with 10% fetal bovine serum (FBS; Intergen Corp.), 100 units/mL of

penicillin, and 100 units/mL streptomycin. LNCaP cells being assayed for luciferase

expression were maintained in 10% strip-serum (charcoal/dextran treated fetal bovine

serum to remove T3, T4, and steroids; Gemini Bioproduct, Inc., Calabasas, CA) RPMI.

The cells were periodically tested for the production of PSA which was consistently above

20   20 ng/mL per day.

Transfections of LNCaP Cells

For transfections, LNCaP cells were plated out at a cell density of 5×10^5 cells per

6-cm culture dish (Falcon, NJ) in complete RPMI. DNAs were introduced into LNCaP

cells after being complexed with a 1:1 molar lipid mixture of N-[1-(2,3-dioleyloxy)propyl-

25   *N,N,N*-trimethylammonium chloride (DOTAP™; Avanti Polar Lipids, AL) and dioleoyl-

phosphatidylethanolamine (DOPE™; Avanti Polar Lipids, AL); DNA/lipid complexes

were prepared in serum-free RPMI at a 2:1 molar ratio. Typically, 8 μg (24.2 nmole) of

DNA was diluted into 200 μL of incomplete RPMI and added dropwise to 50 nmole of

transfecting, lipids in 200 μL of RPMI with gentle vortexing to insure homogenous mixing

30   of components. The DNA/lipid complexes were allowed to anneal at room temperature for

15 minutes prior to their addition to LNCaP cells. Medium was removed from LNCaP

cells and replaced with 1 mL of serum-free RPMI followed by the dropwise addition of DNA/lipid complexes. Cells were incubated with complexes for 4-5 hours at 37°C, 5% CO_2. Medium was removed and cells washed once with PBS. The cells were then trypsinized and resuspended in 10% strip-serum RPMI (phenol red free). Cells were

5   replated into an opaque 96-well tissue culture plate (Falcon, NJ) at a cell density of 40,000 cells/well per 100 μL media and assayed. Varying amounts of drugs (e.g. androgens and anti-androgens) were added 16 hours later and assayed for luciferase activity 32 hours thereafter.

Plaque assays

10   To determine whether the adenoviral constructs described above replicate preferentially in prostate cells, plaque assays were performed. Plaquing efficiency was evaluated in the following cell types: prostate tumor cell lines (LNCaP), breast normal cell line (HBL-100), ovarian tumor cell line (OVCAR-3, SK-OV-3), and human embryonic kidney cells (293). LNCaP cells express both androgen receptor and PSA, while the other

15   cell lines tested do not. 293 cells serve as a positive control for plaquing efficiency, since this cell line expresses Ad5 E1A and E1B proteins. For analyzing constructs comprising a CEA-TRE, cells that allow a CEA-TRE to function, such as NCIH508, LoVo, SW1463, MKN1, MKN28, MKN45 and cells that do not allow such function, such as HuH7, HeLa, PA-1, or G361, are used. For analyzing constructs comprising an AFP-TRE, cell lines

20   which express AFP, such as HepG2 and Hep3B, are compared with cell lines which do not express AFP, including HBL-100, OVCAR-3, and LNCaP. The plaque assay was performed as follows: Confluent cell monolayers were seeded in 6-well dishes eighteen hours before infection. The monolayers were infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the media

25   was removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques were scored two weeks after infection. CN702 has no modifications in its E1 region, but is devoid of E3, and thus serves as a wild type control for left end modifications lacking E3. CN706 demonstrates selective cytotoxicity toward PSA-expressing cells *in vitro* and *in vivo*. Rodriguez et al. (1997) *Cancer Res.* 57:2559-2563.

In vitro characterization of CN787

Various parameters of CN787 were measured, including cell type specificity, plaque size, extracellular virus yield, kinetics of cell killing, and total viral yield per cell.

Cell type specificity of CN787

5   To determine whether adenoviral construct CN787, described above, replicate preferentially in prostate cells, plaque assays were performed as described above. The results are shown in Figure 3. The results are also summarized in Figure 2, where they are expressed as percent of wild-type adenovirus plaque-forming units (PFU) per ml. The average titer of duplicate samples for the viruses tested. The titer for a particular virus in

10   all cell lines was normalized to its titer on 293 cells. Once the titers on a cell type were normalized to 293 cells, the normalized numbers of the recombinant viruses were compared to CN702. A ratio of less than 100 suggests that the virus tested plaques less efficiently than CN702. Conversely, a ratio greater than 100 suggests that the virus plaques more efficiently than CN702. The ratio of specificity of CN787 to that of CN702 is

15   10,000:1.

These results indicate that CN787 exhibits a specificity for prostate cells comparable to that of CN739.

Viral production

20   While CN787 and CN739 demonstrate comparable cell-type specificity, CN787 gave much larger plaques than did CN739, as shown in Figure 4.

One measure of viral production is extracellular virus yield per cell. CN787 and CN739 were plated on LNCaP cells at 2 PFU/cell, and plated at 10^6 cells per well. At various times after infection, the cell supernatant was tested on 293 cells to measure the

25   number of plaque-forming units. The results, presented in Figure 5, show that CN787 gave a significantly higher viral yield than did CN739.

When total virus yield was examined, it was found that, at 96 hours post infection, CN787 yielded at least one log higher number of plaque forming units per cell, as shown in Figure 6. The total viral yield per cell is the extracellular viral yield per cell plus the

30   intracellular viral yield per cell. CN787 yielded more extracellular virus per cell than did CN739, indicating that that E3 mediates a fast release of viruses from the infected cells. The total viral yield per cell from cells infected with CN787 is higher than that of cells

infected with CN739, suggesting that CN787-infected cells produce more viruses. The higher total yield could be due to virus replication/DNA replication, or a higher efficiency of viral DNA packaging.

To examine the kinetics of cell killing by CN787, two different assays for cell viability, the MTT assay and the trypan blue dye exclusion assay, were performed. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay was performed as follows. 293 cells were infected with CN787 or CN739 at a concentration of 2 PFU/cell and plated at a cell density of 10^4 cells per well at various times post-infection. At the indicated times post-infection, 25 µl MTT (Sigma Chemical Co., St. Louis, MO) in PBS at a concentration of 5 mg/ml was added to each well. After 2 hours, lysis buffer (20% sodium dodecyl sulfate in 50:50 dimethyl formamide:double-distilled H_2O, pH 4.7) was added to each well and plates were incubated overnight at 37°C. Plates were read on a microplate reader at 570 nm. Samples were in triplicate. The results, shown in Figure 7, indicate that when cells were infected with CN787, the absorbance dropped sharply after 4 days post infection, and by 7 days post-infection, virtually all cells were killed. In contrast, cells were killed less rapidly by CN739, which is analogous to CN787 but lacks an E3 region.

As a second assay for cell viability, trypan blue exclusion was performed. At various times after infection, a sample of cells 10^6 LNCaP cells was removed. Cells were separated from the medium and trypsinized. Trypan blue (Gibco BRL, Gaithersberg, MD) was added to a final concentration of 0.02%. Cells were counted on a hemacytometer. As with the MTT assay, the trypan blue exclusion assay (Figure 8) showed that a reduction in viability to 50 % viability of LNCaP cells was achieved in 3 days by CN787, compared with 6.5 days by CN739. Thus, by two different assays, LNCaP cells were killed more rapidly by CN787 than by CN739.

In vivo characterization of CN787

An especially useful objective in the development of prostate-specific adenoviral vectors is to treat patients with prostate carcinoma. An initial indicator of the feasibility is to test the vectors using a technique known in the art, such as testing the vectors for cytotoxicity against cancer cells such as tumor xenografts grown subcutaneously in Balb/c nu/nu mice. Mice are given subcutaneous injections with 1 X 10^7 carcinoma cells, such as

LNCaP or HepG2, in PBS. Tumor cells can be tested for secretion of a tumor-specific product, such as, for example, PSA or AFP, by assaying for the product in serum using standard assays (for example, ELISA). Alternatively, as described below, tumor volume can be measured.

5   LNCaP nude mouse xenografts were challenged with a single intratumoral dose (1 X 10^4 particles/mm^3 tumor) of either CN787, a vector containing the E3 gene, or CN739, a vector lacking the gene. A third group of tumors was treated with buffer alone. The tumors were monitored weekly for six weeks and their relative volume was graphed against time. The results are shown in Figure 9. Error bars represent the standard error for each

10   sample group. The initial average tumor volume for CN787 treated animals (n =7) was 270 mm^3, 295 mm^3 for CN739 treated (n = 7), and 270 mm^3 for buffer treated (n =7). The data suggest that CN787 kills tumor cells more effectively than CN739. On average, tumors challenged with CN739 remained the same size throughout the course of the experiments. Those treated with vehicle alone more than doubled in size. Tumors challenged with

15   CN787 were reduced in size by 40% by week 5. The Students T-test indicates that the difference in tumor size between CN787 and CN739 treated tumors was statistically significant from week 3 through the end of the experiment (p = 0.00209).

Figure 10 presents the results of regimen optimization efforts. LNCaP nude mouse xenografts were challenged with 5 x 10^{10} CN787 viral particles per day for 5 days (total of

20   2.5 x 10^{10} particles CN787) (p = 1.47 x 10^{-5}) or 1 x 10^{11} CN787 viral particles per day for 3 days (total of 3 x 10^{11} CN787 viral particles)(p = 9.2 x 10^{-7}).

EXAMPLE 3

In vitro and in vivo characterization of CN790, an E3-containing adenoviral construct comprising AFP-TREs driving expression of E1A and E1B

25

In vitro characterization of CN790

To determine whether adenoviral construct CN790, described above, replicates preferentially in liver cells, plaque assays were performed as described in Example 2. The results, shown in Figure 12, are expressed as percent of wild-type (CN702) adenovirus

30   plaque-forming units (PFU) per ml. The average titer of duplicate samples for the viruses tested. The titer for a particular virus in all cell lines was normalized to its titer on 293

cells. Once the titers on a cell type were normalized to 293 cells, the normalized numbers of the recombinant viruses were compared to CN702. A ratio of less than 100 suggests that the virus tested plaques less efficiently than CN702. Conversely, a ratio greater than 100 suggests that the virus plaques more efficiently than CN702.

5   CN790 showed a plaquing efficiency comparable to that of CN733 on HepG2 and Hep3B cells. The results indicate that CN790 demonstrates specificity for hepatic cells comparable to that of CN733.

       Virus yield assay. Twenty-four well dishes (Falcon) were seeded with 200,000

10   cells per well of Hep3B, HepG2, LNCaP, OVCAR-3, or SK-Hep-1 cells 24 hr prior to infection. Cells were infected at an MOI of 2 PFU/cell for three hours in serum-free media. After the infection was complete, the virus containing media was removed, monolayers were washed three times with PBS, and one ml of complete media was added to each well. 72 hrs post infection, cells were scraped into the culture medium and lysed by three cycles

15   of freeze-thaw. Two independent infections of each virus cell-combination were titered in duplicate on 293 cells.

       Results. The addition of the Ad E3 region did not alter virus cell tropism. Cell lines were infected with either CN790 or CN802 (wt Ad5) at a multiplicity of infection (MOI) of 2 PFU/cell. Progeny virus yield was determined 72 hrs after infection by plaque

20   assay on 293 cells (Figure 32). In AFP$^+$ cells (Hep3B, HepG2) CN790 replicated similarly to the wild-type control; only a 2-6 fold difference in virus yield was observed. However, CN790 replicated poorly in all AFP$^-$ cells tested (LNCaP, OVCAR-3, SK-Hep-1), producing a 10^5- to 10^6-fold lower virus yield than CN802. These data indicate that CN790 replicates efficiently in AFP$^+$ cells, but is significantly attenuated in AFP$^-$ cells.

25   Comparison of CN733 and CN790 in the one-step growth curve in Hep3B and OVCAR-3 cells showed CN790 had the same level of specificity as CN733.

       Cytopathic effect of CN790

       Cytopathic effect (CPE) assay was carried out to visually assess the specificity of

30   CN790 cytolytic activity. Six well dishes (Falcon) were seeded at 300,000 cells per well with HepG2, Hep3B, Huh-7, LNCaP, OVCAR-3 and SK-Hep-1 cells 24 hr prior to

infection. Cells were infected with CN790 or CN802 at an MOI of 10 PFU/cell, except for SK-Hep-1 cells, which were infected at an MOI of 20 PFU/cell. Virus adsorption was for three hours in 1 ml of serum-free media. Following adsorption, the virus containing media was removed and replaced with 3 ml of RPMI, 10% FBS. Seven days post infection, cells
5   were stained with crystal violet.

Three AFP+ cell lines (Hep3B, HepG2, Huh-7) and three AFP- cell lines (LNCaP, OVCAR-3, SK-Hep-1) were mock infected (control) or infected with either CN790 or CN802 (wt Ad5) at an MOI of 10 PFU/cell. SK-Hep-1 cells were infected at an MOI of 20 PFU/cell. Cytopathic effect (CPE) seven days post-infection was monitored by staining
10 remaining cells with crystal violet. The results are shown in Figure 33.

The results indicate that CN790 caused complete CPE in the three AFP expressing cells, but caused little CPE in the three AFP- cells tested, including the AFP- hepatoma line SK-Hep-1. In contrast, wt Ad5 caused significant CPE in all cell lines tested regardless of AFP status. Thus, the cytolytic activity of CN790 is specific for AFP+ cell lines and
15 CN790 causes little toxicity to AFP-cells.

In vivo characterization of CN790

The effect of CN790 on HepG2 tumors was tested using the tumor xenograft assay system. Six to eight week old athymic Balb/c *nu/nu* mice were obtained from Simonson Laboratories (Gilroy, CA). Xenografts were established by injecting 1-3 x 10^6 Hep3B or
20 HepG2 cells subcutaneously near the small of the back suspended in 100 μl of RPMI. When tumors reached between 200 μl and 300 μl, mice were randomized and treated with 100 μl of CN790 by tail vein injection. Two treatment regimens were tested. First, Hep3B tumors were treated with 4 x 10^9 PFU of CN790 or vehicle alone (PBS + 10% glycerol) on days 1, 4 and 8. Second, HepG2 tumors were treated with 5 x 10^8 PFU of CN790 or
25 vehicle on days 1, 2, 3, 4, 5, 8, 9, 10, 11, and 12. Tumors were measured in two dimensions by external caliper and volume was estimated by the formula [length (mm) x width $(mm)^2$]/2. Animals were sacrificed when tumor burdens became excessive. Serum was harvested weekly by retro-orbital bleed.

HepG2 tumors. On days 1-5 and 8-12 after introducing the HepG2 cells into the
30 mice, CN733, CN739, or CN790, at a concentration of 1×10^{10} particles in a volume of 50

µl, or, as a control, vehicle alone, was injected into the mice intravenously. There were 10

mice per test group and in the vehicle-only control group. The results are shown in Figure

13. On average, tumors treated with CN733 or CN790 four weeks after treatment were 857

mm^3 and 256 mm^3, respectively. Tumors treated with either CN739 or vehicle were 1521

5   mm^3 and 1601 mm^3, respectively. Even though both CN733 and CN790 were able to delay

tumor progression, CN790 produced a greater tumor response. Both CN733- and CN790-

treated tumors increased in size from four to six weeks after the start of treatment. As

expected, CN739, a prostate-specific virus, had no effect on tumor growth. CN739-treated

tumors, unlike CN733- and CN790-treated tumors, appeared well vascularized. In contrast,

10   CN733- and CN790-treated tumors had a mottled appearance indicative of tumor necrosis.

Hep3B tumors. Subcutaneous Hep3B xenografts in nude mice were treated with

intravenously administered CN790. Mice were treated with either 4 x 10^9 PFU of CN790

(particle/PFU = 20/1) or vehicle alone on days 1, 4, and 8 via tail vein injection. There

were 7 mice per test group and 8 in the vehicle-only control group. Tumor size and the

15   concentration of serum AFP were monitored weekly. Control buffer treated mice were

sacrificed at four weeks due to excessive tumor burden. Average tumor volumes and

serum AFP levels were normalized to 100% at day 1. The results are shown in Figure 34A.

The data demonstrate significant tumor killing from CN790. At day 29, the average

relative tumor volume of CN790 treated mice was 142%, whereas vehicle treated was

20   899%. At day 43, one mouse was tumor-free and two others exhibited tumor regression

out of seven total animals. Statistical analysis of the results was conducted using the

unpaired, two-tailed t-test. The differences in mean relative tumor volumes between

CN790 treated and buffer treated tumors are significant at days 15, 22 and 29 ($p < 0.05$).

Serum AFP levels in mice treated with CN790

25   Besides tumor volume, another measure of the efficacy of treatment of tumors is

serum concentration of a tumor marker. Serum AFP levels were measured at various times

after treatment with adenoviral vectors. The results are shown in Figure 14. HepG2 mice

(i.e., mice harboring a HepG2 xenograft) treated as described above with either CN733 or

CN790 had average serum AFP concentrations of 214 µg/ml and 79 µg/ml, respectively, at

30   four weeks after the start of treatment. In contrast, mice treated with either vehicle or

CN739 had serum AFP concentrations of 443 µg/ml and 619 µg/ml, respectively. In

general, CN733 and CN790 slowed the rise in serum AFP concentration compared to CN739 or buffer. The difference in mean AFP concentration between vehicle-treated and CN733-treated and between vehicle-treated and CN790-treated is significant beginning at week 4.

5   The levels of serum AFP in treated Hep3B mice provide additional evidence of CN790 antitumor activity. At day 29, CN790 treated mice had an average serum AFP concentration of 269%, and vehicle treated mice were 2161%. At day 43, the concentration in CN790 treated mice was 110%. Three mice had decreased serum AFP levels after dosing (Figure 34B). Statistical analysis of the results was conducted using the unpaired,

10   two-tailed t-test. The differences in mean serum AFP concentrations between CN790 treated and buffer treated tumors are significant at days 15, 22 and 29 ($p < 0.05$).

In summary, intravenously administered CN790 is more effective at delaying the growth of HepG2 tumors and slowing the rise of serum AFP levels in a nude mouse model for primary liver cancer, when compared to $E3^-$ CN733. Furthermore, CN790 not only

15   delayed the growth of Hep3B tumors but eradicated the tumor in one of the mice tested. The greater anti-tumor effective, $E3^+$, CN790 indicates that adenoviral vectors that contain an E3 region or portion of E3 are more cytotoxic than those that are deleted for E3. Cells infected with E3-containing virus also release more progeny virus and can be lysed sooner than cells infected with E3-deleted viruses.

20

Mechanism of Tumor Cytolysis by CN790

Immunohistochemistry

Hep3B tumors were established in _nu/nu_ mice. Three mice treated with buffer and

25   three mice treated with CN790 (4×10^9 PFU/dose) on days 1, 4, and 8 were sacrificed on day 9. Tumors were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned using standard procedures. To detect adenovirus, tissue sections were blocked with normal rabbit serum (Biogenex, San Carlos, CA) for 20 min and incubated with goat anti-Ad antibody (Biodesign International, Kennebunkport, ME) diluted 1:200 in PBS for

30   30 min. Alkaline phosphatase staining was performed using Super Sensitive streptaviden-biotin alkaline phosphatase reagents and Fast Red chromogen (Biogenex) as suggested by

the manufacturer. Sections were counterstained with Gill's hematoxylin and mounted with Gel Mount (Biomedia, Foster City, CA) aqueous mounting media.

Apoptosis Assay

Apoptotic cells were detected using TdT labeling (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, Intergen, Purchase, NY). Sections were pretreated with 20 µg/ml of protinase K for 15 min, counterstained with methyl green and mounted with Permount (Fisher). To evaluate the number of apoptotic cells in virus-treated and un-treated control tumors, the tumors were sectioned through their largest diameter and slices were prepared from each half of the resected tumor. The apoptotic index was estimated by the number of positive cells/100X field, eight sections per tumor. A minimum of five 100X fields were counted for each section. The mean of the sections was used for the apoptotic assay, and representative sections presented.

Results. To investigate mechanisms of CN790 tumor destruction, Ad5 hexon staining for virus replication and the apoptotic index by TdT labeling of treated tumors was determined. Virus replication in Hep3B tumors was confirmed by immunohistochemical staining of tumor sections using polyclonal antibodies to Ad5 hexon. Ad5 hexon is not found immediately or several days after intravenous virus administration. However, Ad5 hexon can be detected by immunohistochemistry late in the virus replication cycle. Figure 35A shows Hep3B xenograft tumors stained for Ad5 hexon 9 days following treatment intravenously with CN790. While positively stained cells were visible throughout the tumor, infected cells were predominantly located near the tumor periphery. No adenovirus was detected in vehicle treated tumors.

To estimate CN790 induced apoptosis, three CN790 treated and three control tumors were analyzed nine days after initial dosing (Figure 35B). Treated tumors had a 70% higher apoptotic index than control tumors. Because apoptosis is short-lived, even modest increases in the apoptotic index of a tumor may result in a significant antitumor effect. Bursch et al. (1992) *Trends Pharmacological Science* 13:245-251; and Bursch et al. (1990) *Carcinogenesis* 11:847-853. Apoptotic bodies were observed both adjacent to infected cells and uninfected areas, indicating that apoptosis may augment virus mediated killing. Thus, the immunohistochemical analysis of CN790 treated tumors suggests that

both virus replication-dependent cytolysis and apoptosis contribute to the CN790 antitumor effect.

Furthermore, CN790 caused plaques that were estimated to be 5 times larger than those lysed by CN733 on 293 cells 6 days after infection (Figure 31), indicating that CN790 possesses higher potency to induce apoptosis than CN733.

In summary, CN790 grows at least 100,000 fold less efficiently than the wild-type E1 viruses CN702 and CN802 in AFP⁻ cells (OVCAR-3 cells). Most importantly, intravenous administration of CN790 inhibited the growth of HCC xenografts and stabilized the concentration of serum tumor marker AFP to a greater extent than CN733. Immunohistochemical analysis of treated tumors confirmed that systemically administered CN790 productively infected the tumor mass and caused tumor killing. Furthermore, our analysis revealed that treated tumors had a higher apoptotic index than control tumors, suggesting that virus replication-dependent cytolysis and apoptosis were mechanisms of tumor destruction.

EXAMPLE 4

In vitro characterization of E3-containing adenoviral constructs comprising a CEA-TRE driving expression of E1A

To examine the cell-type specificity of CN802, CN799, and CN798, various cell lines were infected at a concentration of 2 PFU per cell with the constructs. The number of plaque-forming units was determined 48 hours post-infection, as described above. As shown in Figure 17, CN798, which contains an E3 region, plaqued efficiently on LoVo, and SW1463 cells, which synthesize CEA, but inefficiently on PA-1 and G361 cells, which do not. In contrast, CN799 and CN802 plaqued efficiently on all cell lines tested. Thus, CN798 appears to specific for CEA-expressing cell lines.

As another measure of cell specificity, LoVo, PA-1, and G361 cells were infected with CN802 or CN798, and viral DNA was measured. Equal amounts of cellular DNA were added to wells and quantitated by a slot blot assay. As shown in Figure 18, viral DNA was undetectable in PA-1 or G361 cells infected with CN798, while approximately 0.4 ng CN798 DNA was measured in infected LoVo cells. In contrast, CN802 viral DNA was detected in all four cell lines infected with this construct.

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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention,

5   which is delineated by the appended claims.

CLAIMS

What is claimed is:

1. An adenovirus vector comprising

5   (a) an adenovirus gene under transcriptional control of a target cell-specific transcriptional regulatory element (TRE); and

              (b) an E3 sequence.

2. The adenovirus vector of claim 1, wherein the adenovirus gene is essential for

10   replication.

3. The adenovirus vector of claim 2, wherein the adenovirus gene is an early gene.

4. The adenovirus vector of claim 3, wherein the early gene is E1A.

15

5. The adenovirus vector of claim 3, wherein the early gene is E1B.

6. The adenovirus vector of claim 3, wherein the early gene is E2.

20   7. The adenovirus vector of claim 3, wherein the early gene is E4.

8. The adenovirus vector of claim 1, wherein the target cell-specific TRE is cell type specific.

25   9. The adenovirus vector of claim 8, wherein the cell type-specific TRE is prostate cell specific.

10. The adenovirus vector of claim 9, wherein the TRE is a PSA-TRE.

30   11. The adenovirus vector of claim 9, wherein the TRE is a PB-TRE.

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12. The adenovirus vector of claim 8, wherein the cell type-specific TRE is liver cell specific.

13. The adenovirus vector of claim 12, wherein the TRE is an AFP-TRE.

14. The adenovirus vector of claim 8, wherein the cell type-specific TRE is breast cancer cell-specific.

15. The adenovirus vector of claim 14, wherein the TRE is a MUC1-TRE.

16. The adenovirus vector of claim 14, wherein the TRE is a CEA-TRE.

17. The adenovirus vector of claim 16, wherein the CEA-TRE comprises nucleotides -6072 to -3815 to -300 to +70 relative to the CEA gene transcription start site.

18. The adenovirus vector of claim 8, wherein the cell type-specific TRE is colon cancer cell-specific.

19. The adenvoirus vector of claim 8, wherein the TRE is an hKLK2-TRE.

20. The adenovirus vector of claim 1, wherein the target cell-specific TRE is cell status specific.

21. The adenovirus vector of claim 20, wherein the cell status-specific TRE comprises a hypoxia-responsive element.

22. The adenovirus of claim 1 comprising an adenoviral gene under transcriptional control of a cell status-specific TRE and a cell type-specific TRE.

23. The adenovirus of claim 22, wherein the cell status-specific TRE comprises a hypoxia-responsive element (HRE) and the cell type-specific TRE is a prostate-specific antigen TRE (PSA-TRE).

5   24. The adenovirus vector of claim 23, wherein the HRE comprises the nucleotide sequence depicted in SEQ ID NO:9.

25. The adenovirus vector of claim 24, wherein the PSA-TRE comprises nucleotides -5322 to -3738 and -541 to +12 relative to the PSA gene transcription start site.

10

26. The adenovirus vector of claim 1, wherein the E3 sequence is under transcriptional control of a target cell-specific TRE.

27. An adenovirus vector comprising (a) an E3 sequence; and (b) a first adenovirus
15   gene under transcriptional control of a first target cell-specific transcriptional response element (TRE) and a second gene under transcriptional control of a second target cell-specific TRE.

28. The adenovirus vector of claim 27, wherein the first adenovirus gene is essential
20   for adenovirus replication.

29. The adenovirus vector of claim 27, wherein the gene essential for replication is an adenovirus early gene.

25   30. The adenovirus vector of claim 29, wherein the early gene is E1A.

31. The adenovirus vector of claim 29, wherein the early is E1B.

32. The adenovirus vector of claim 29, wherein the early gene is E2.

30

33. The adenovirus vector of claim 29, wherein the early gene is E4.

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34. The adenovirus vector of claim 27, wherein the first and second genes are essential for adenovirus replication.

35. The adenovirus vector of claim 34, wherein the first and second genes are adenovirus early genes.

36. The adenovirus vector of claim 27, wherein the second gene is a transgene.

37. The adenovirus vector of claim 36, wherein said transgene is a cytotoxic gene.

38. The adenovirus vector of claim 27, wherein the first and second target cell-specific TREs are cell-type specific and functional in the same cell.

39. The adenovirus vector of claim 29, wherein the first and second target cell-specific TREs are prostate cell-specific.

40. The adenovirus vector of claim 39, wherein the first TRE is a *PB*-TRE.

41. The adenovirus vector of claim 39, wherein the second TRE is a *PSA*-TRE.

42. The adenovirus vector of claim 39, wherein the first TRE is a PB-TRE and the second TRE is a PSA-TRE.

43. The adenovirus vector of claim 42, wherein the *PB*-TRE comprises nucleotides -426 to +28 relative to the rat probasin gene transcription start site, and the *PSA*-TRE comprises nucleotides -5322 to -3738 and -541 to +12 relative to the *PSA* gene transcription start site.

44. The adenovirus vector of claim 27, wherein the first and second target cell-specific TREs are liver cell-specific.

45. The adenovirus vector of claim 44, wherein the first and second TREs are *AFP*-TREs.

46. The adenovirus vector of claim 45, wherein the *AFP*-TREs comprise nucleotides -3954 to -3335 and -174 to +29 relative to the *AFP* gene transcription start site.

47. The adenovirus vector of claim 46, wherein the first and second TREs are colon cancer cell-specific.

48. The adenovirus vector of claim 47, wherein the first and second TREs are *CEA*-TREs.

49. An isolated host cell comprising the adenovirus vector of claim 1.

50. An isolated host cell comprising the adenovirus vector of claim 27.

51. A composition comprising the adenovirus vector of claim 1.

52. A composition comprising the adenovirus vector of claim 27.

53. A method for conferring selective cytotoxicity on a cell which allows a target cell-specific TRE to function, comprising contacting said cell with an adenovirus vector of claim 2, wherein the adenovirus vector enters the cell.

54. A method according to claim 53, wherein cell is a mammalian cell.

55. A method according to claim 54, wherein the mammalian cell is a prostate cell.

56. A method according to claim 54, wherein the mammalian cell is a liver cell.

57. A method according to claim 54, wherein the mammalian cell is a breast cancer cell.

58. A method according to claim 55, wherein the mammalian cell is a colon cancer cell.

59. A method for propagating an adenovirus vector of claim 1, said method comprising combining an adenovirus vector of claim 1 with cells which allow function of the target cell-specific TRE, whereby said adenovirus is propagated.

60. A method according to claim 59, wherein the cells are mammalian cells.

61. A method for suppressing tumor growth comprising contacting a tumor cell with an adenovirus vector according to claim 2 such that the adenovirus vector is introduced into the tumor cell.

62. A method according to claim 61, wherein the tumor cell is a mammalian cell.

63. A method according to claim 62, wherein the mammalian cell is a prostate cell.

64. A method according to claim 61, wherein the mammalian cell is a liver cell.

65. A method according to claim 61, wherein the mammalian cell is a breast cancer cell.

66. A method according to claim 61, wherein the mammalian cell is a colon cancer cell.

67. A method for modifying the genotype of a target cell comprising contacting the target cell with the adenovirus vector according claim 1 such that the adenovirus vector is introduced into the target cell.

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68. A replication competent adenovirus vector comprising an E3 sequence under transcriptional control of a target cell-specific TRE.

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Region E3 of Adenovirus
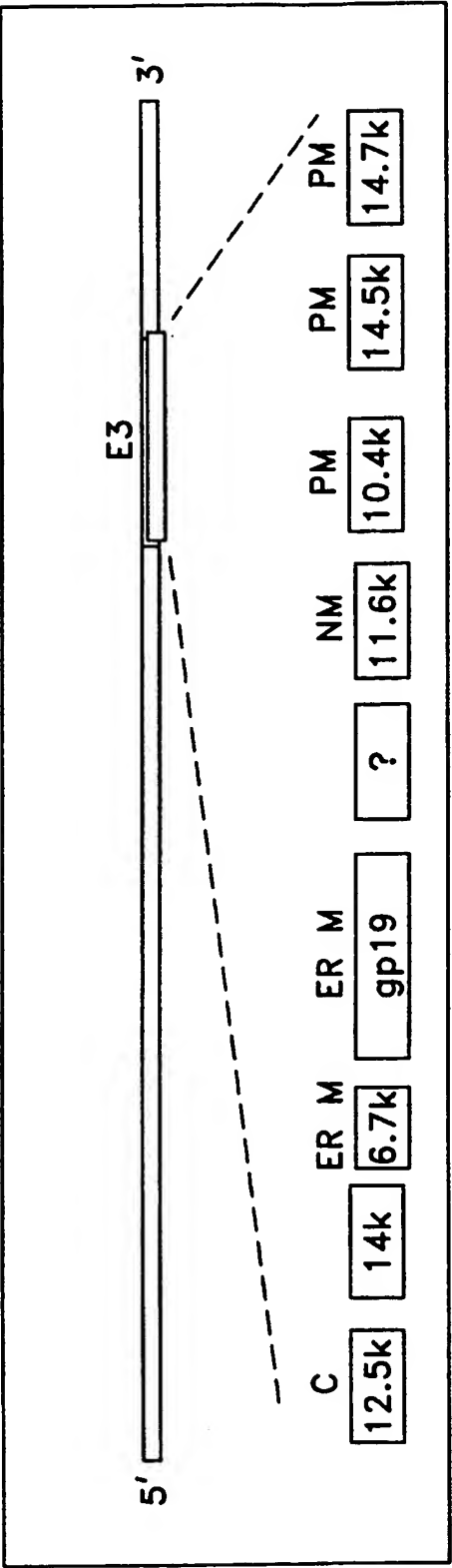
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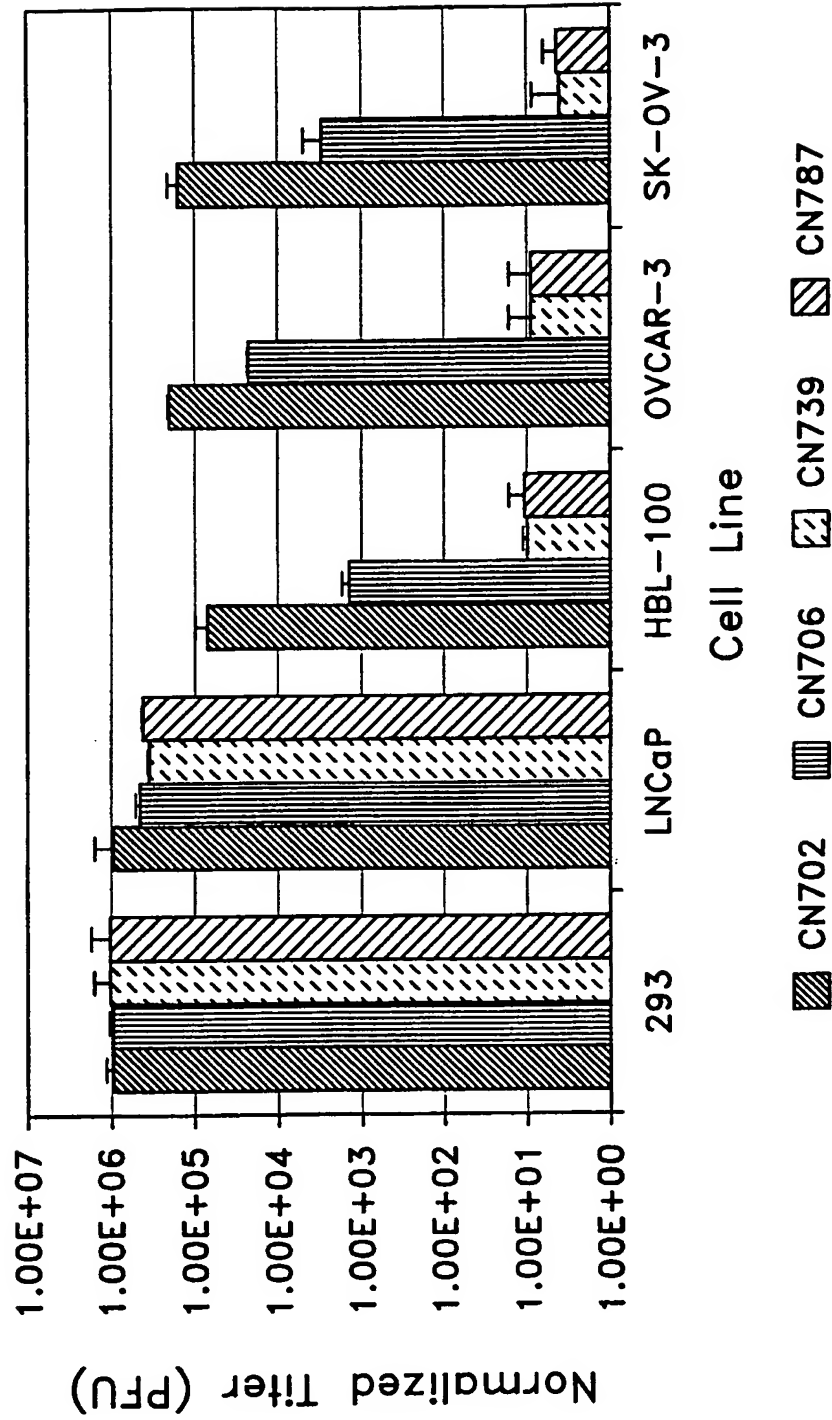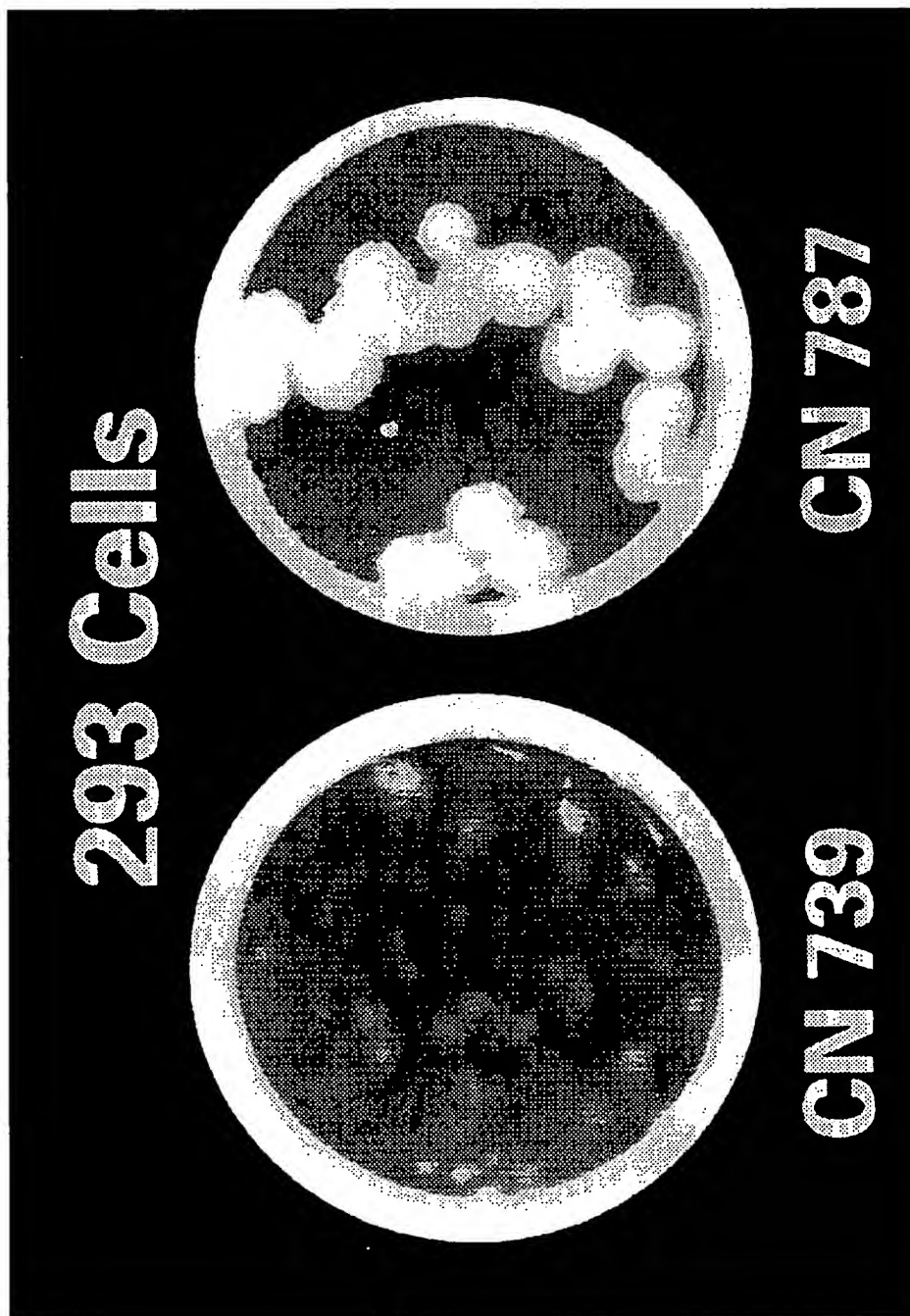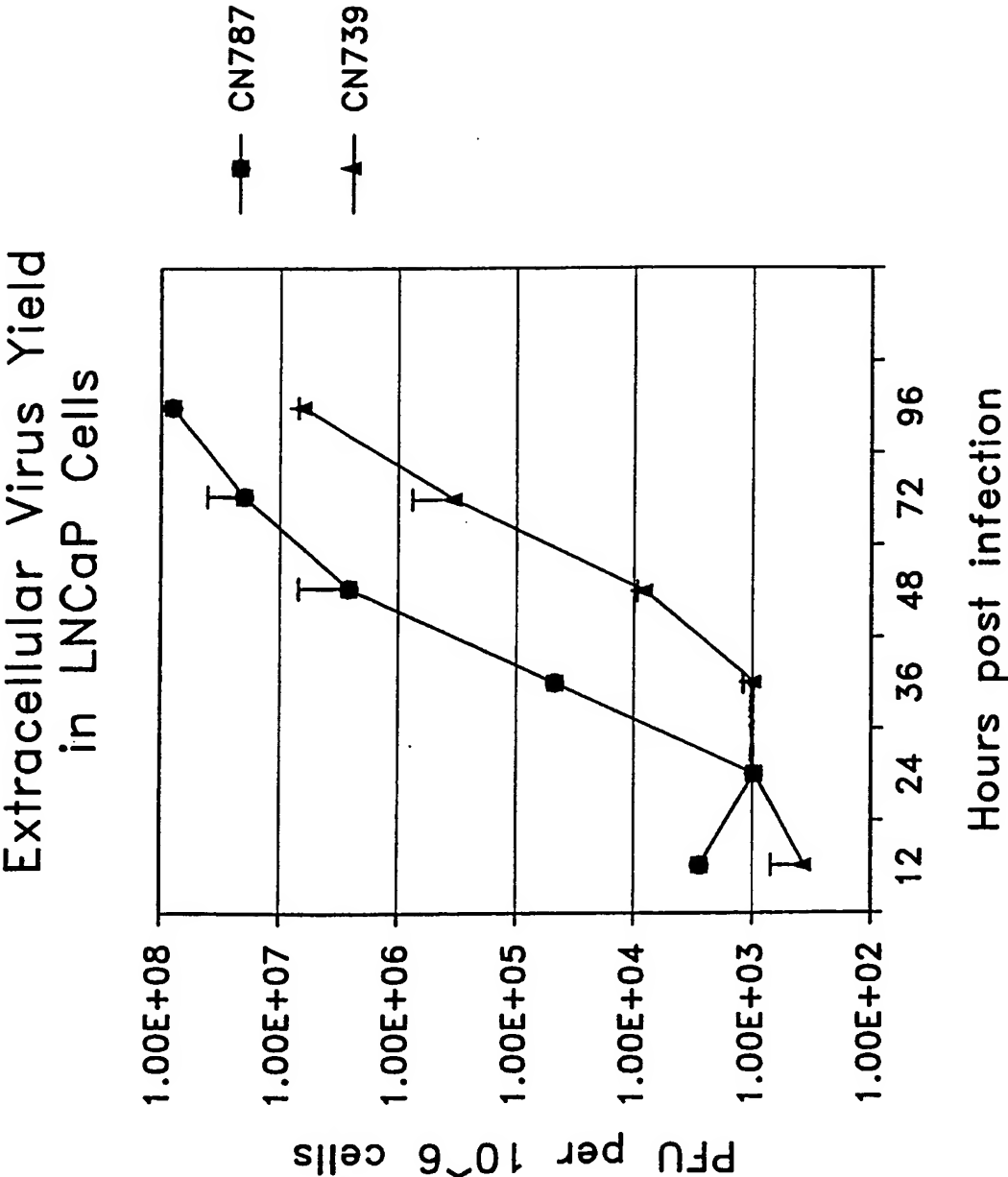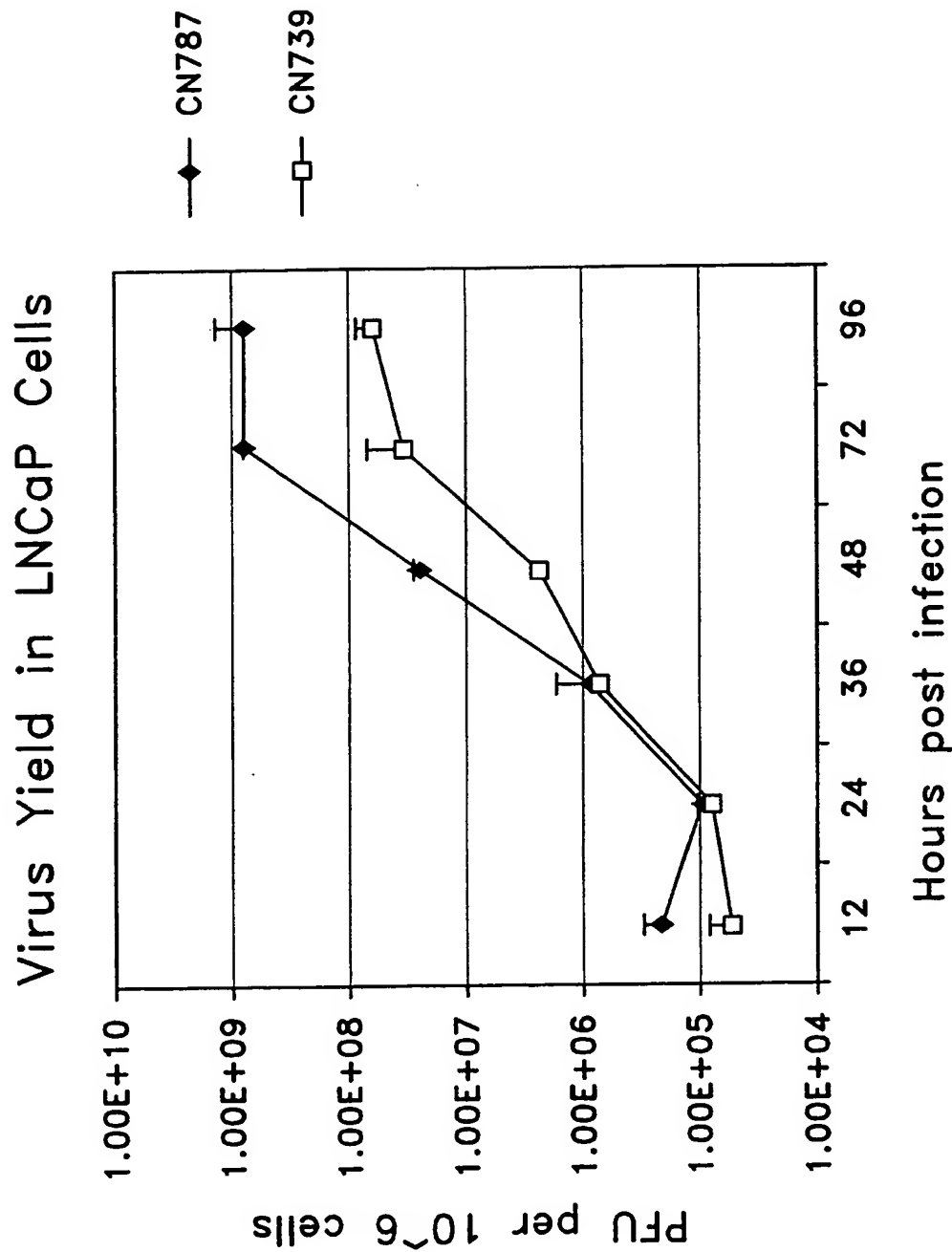
FIGURE 2

FIGURE 3

Figure 4

FIGURE 5

FIGURE 6
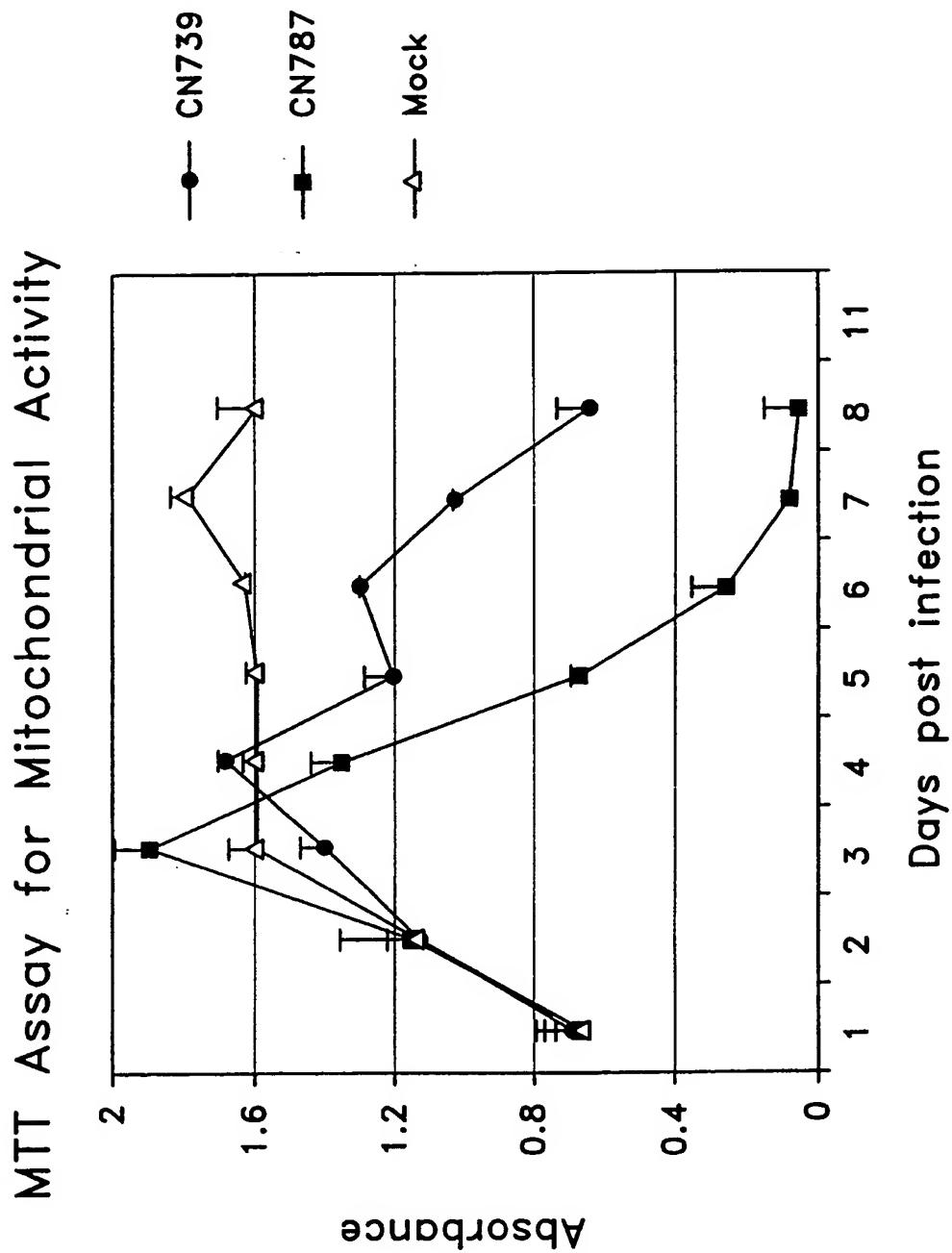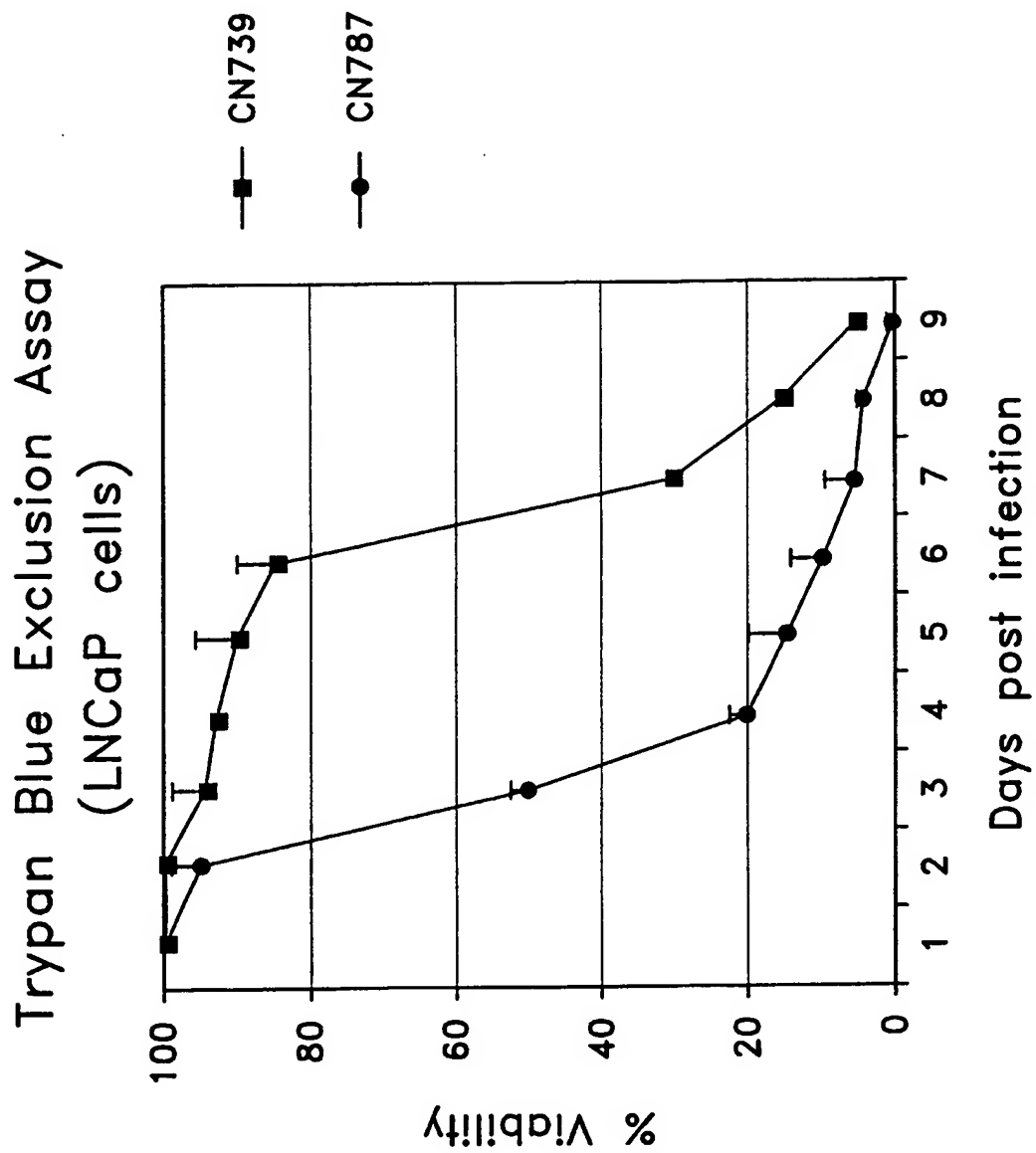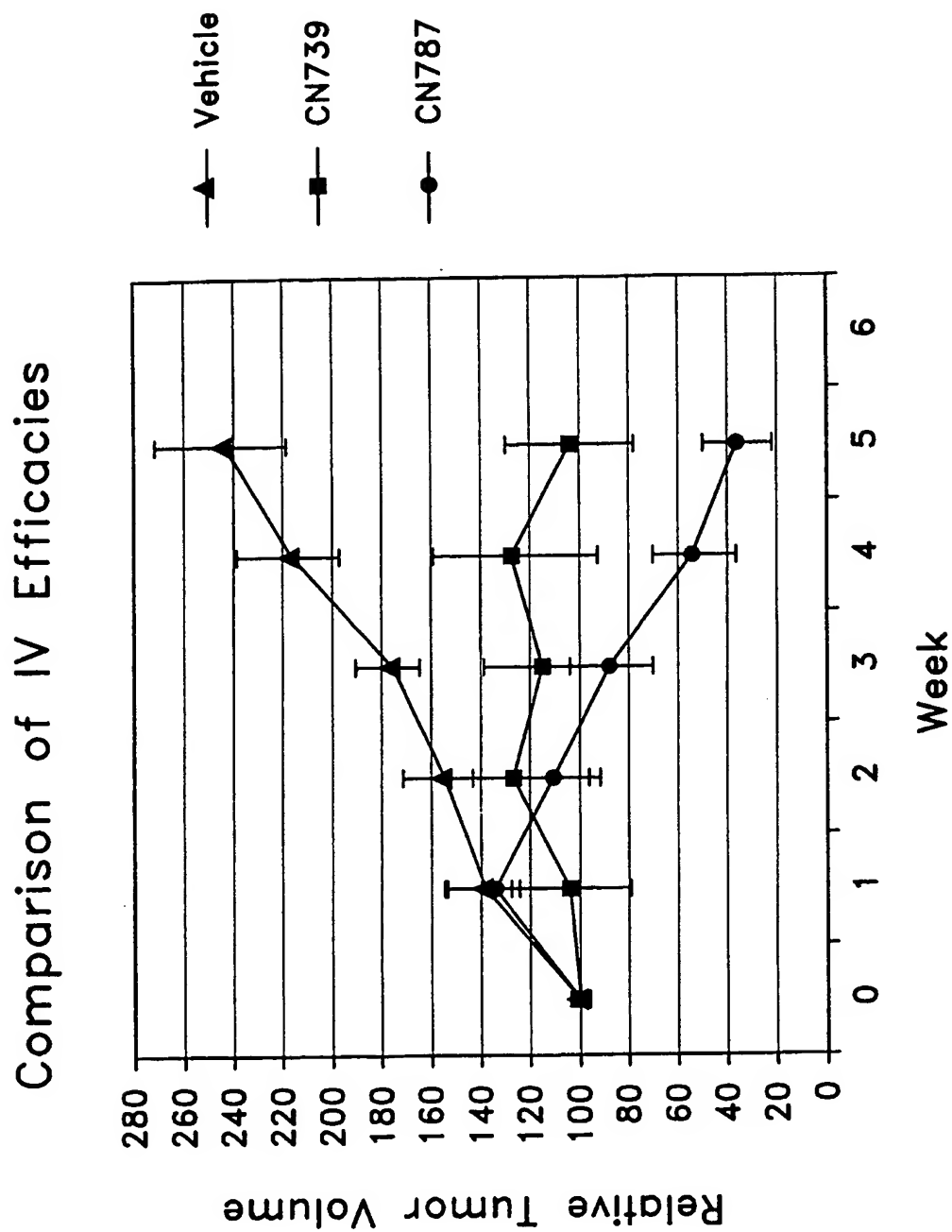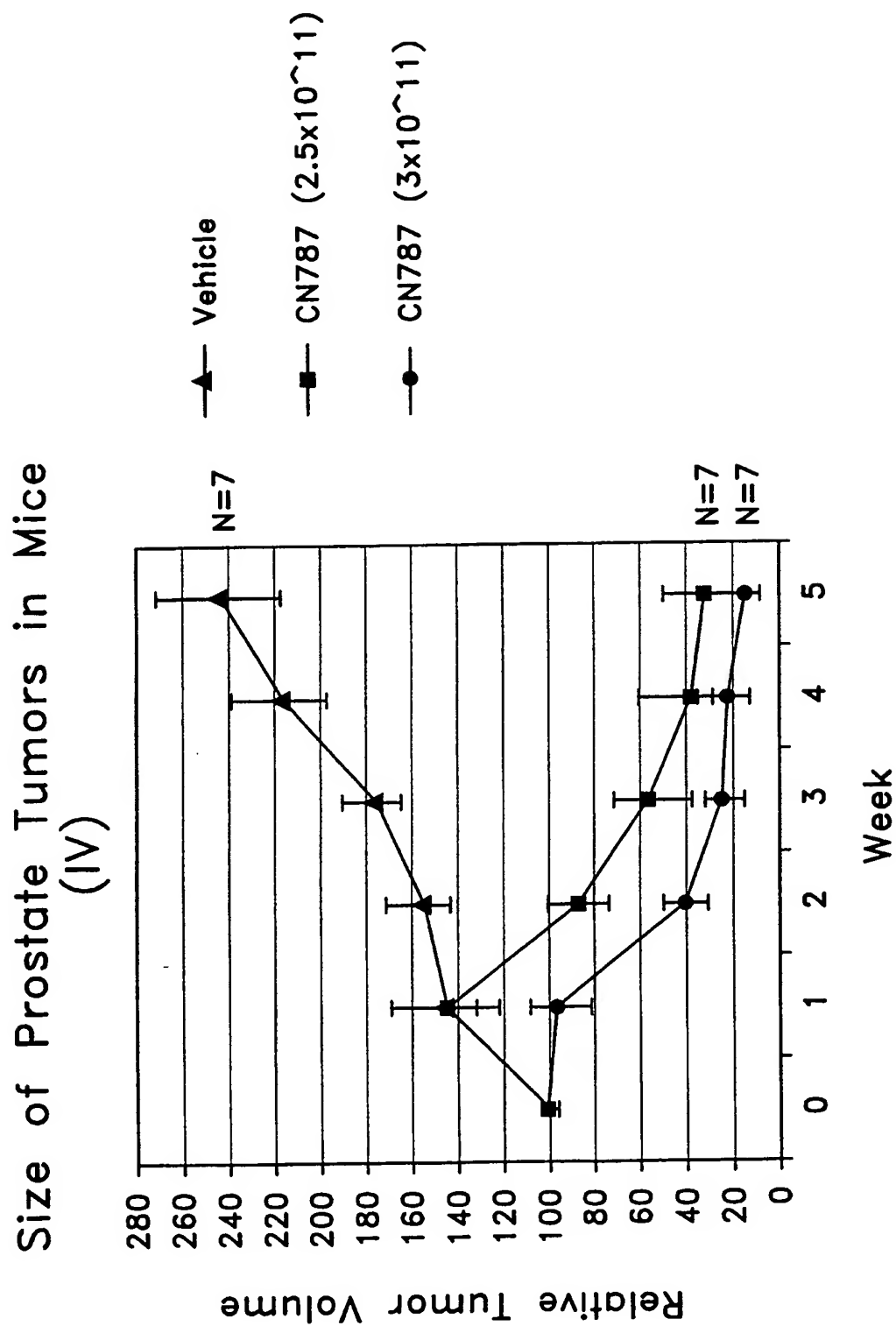
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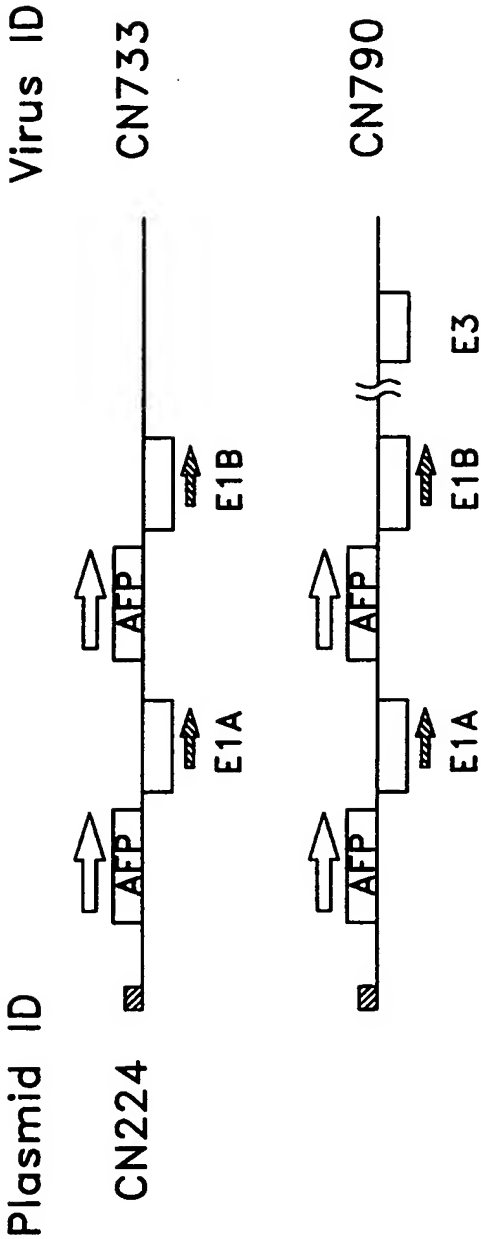
FIGURE 8

FIGURE 9

FIGURE 10

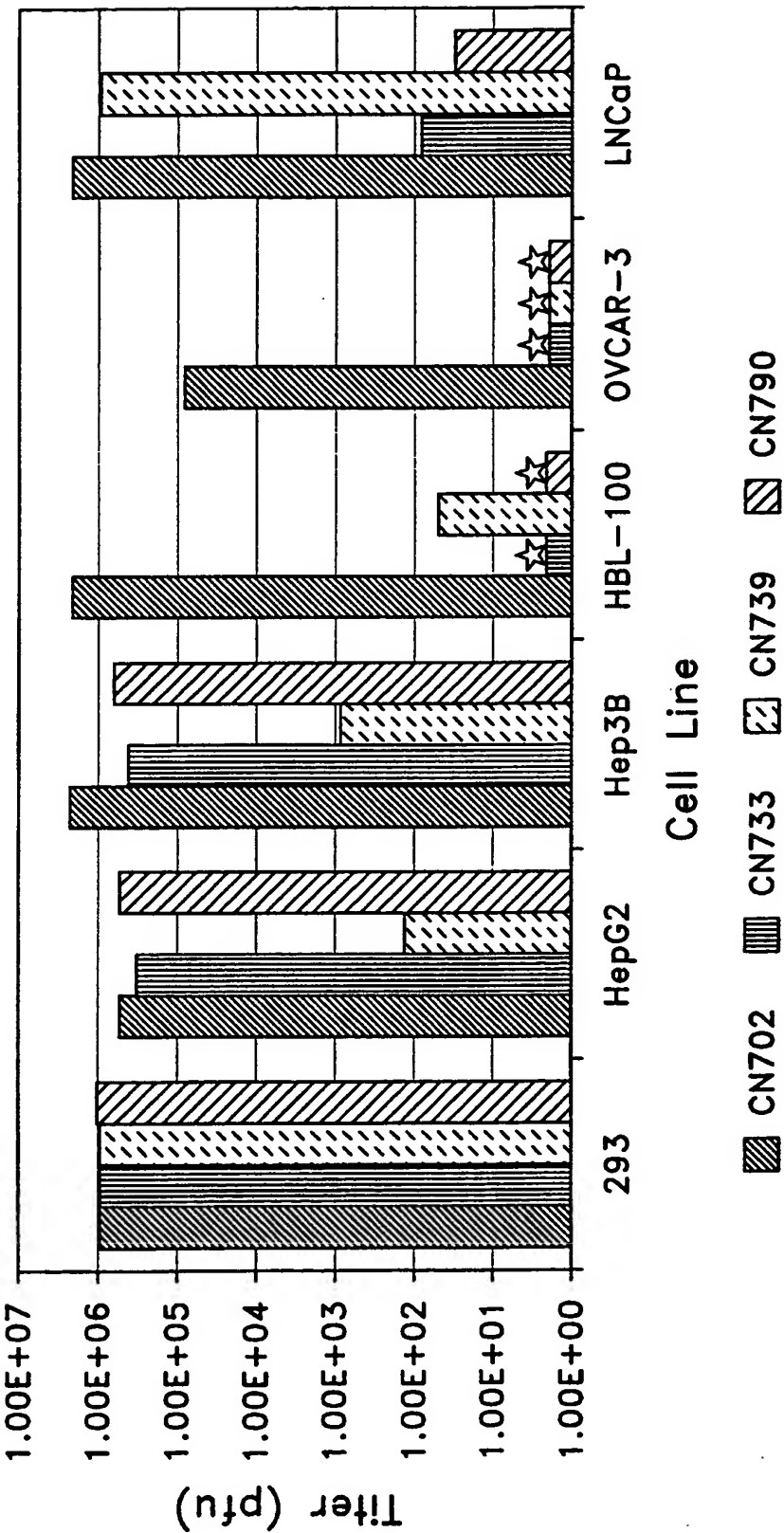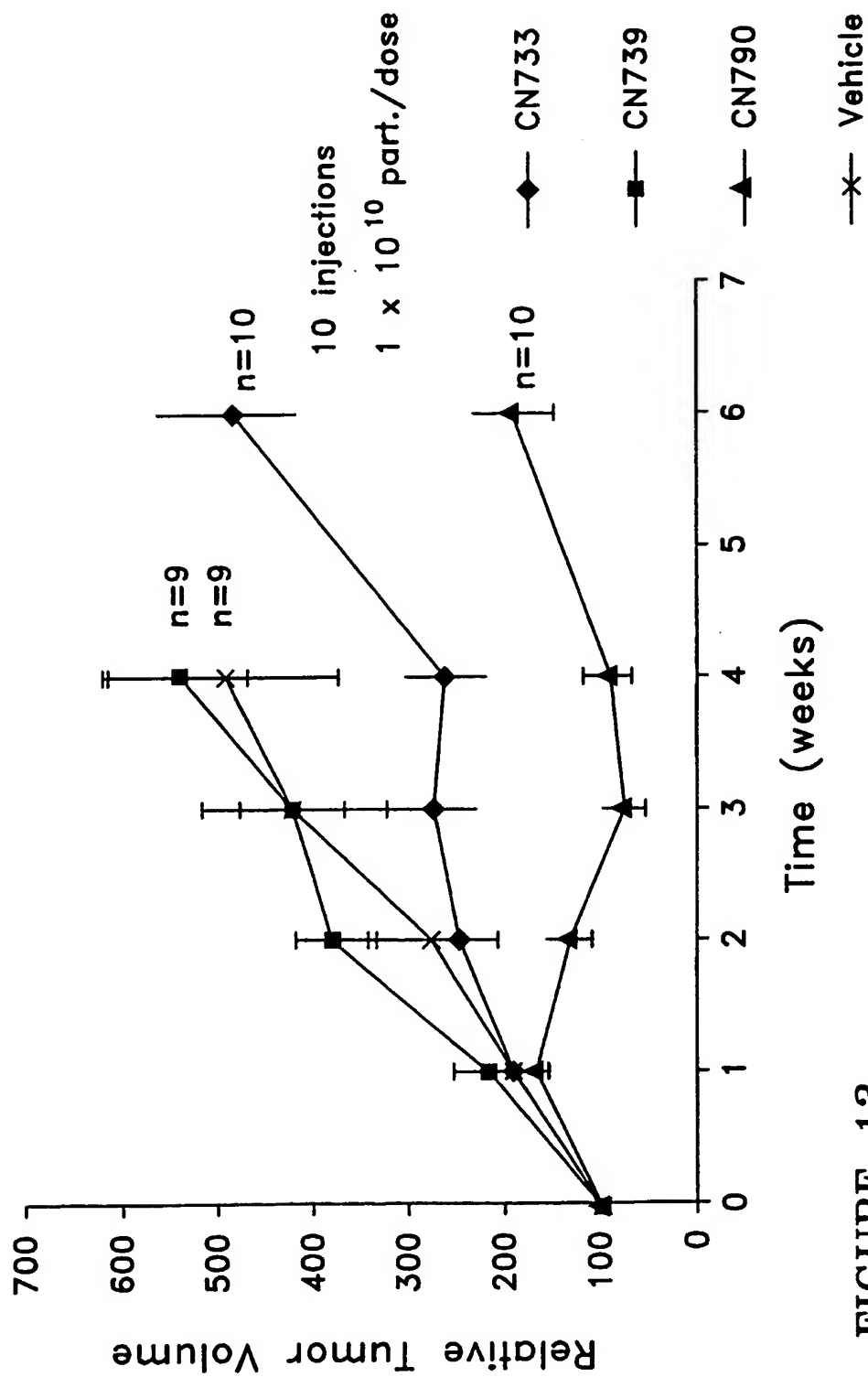FIGURE 11
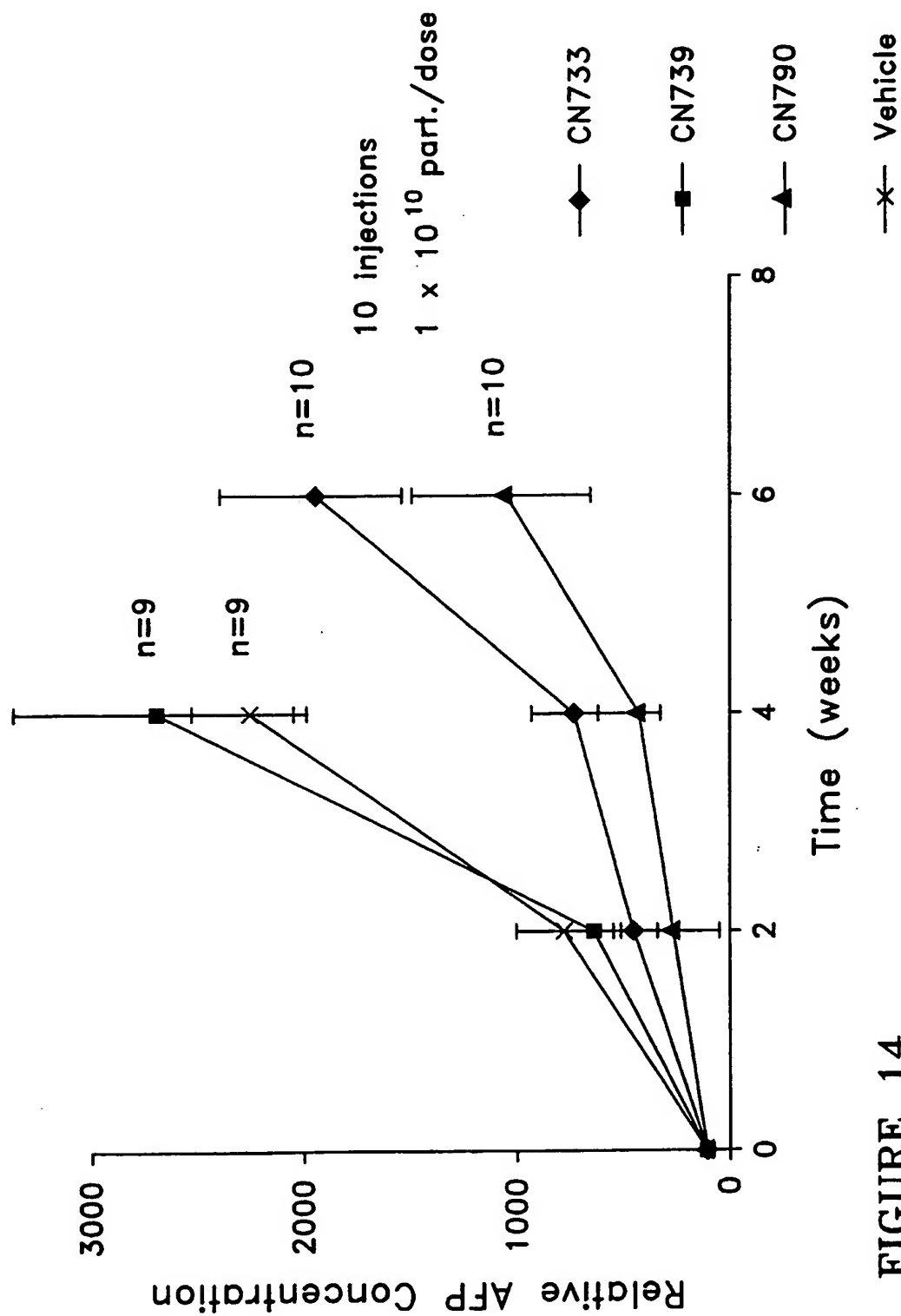
FIGURE 12

FIGURE 13

FIGURE 14

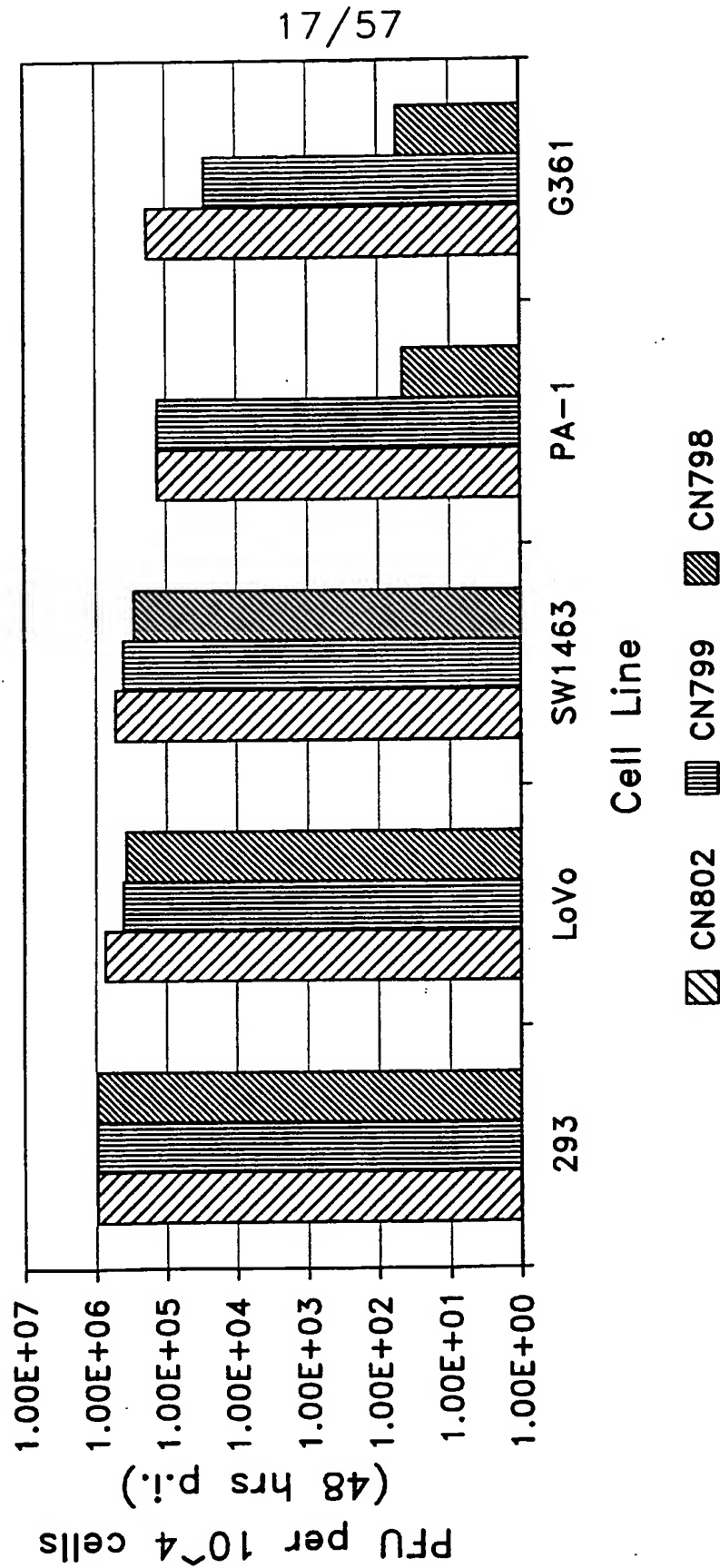CEA Transcription Response Element
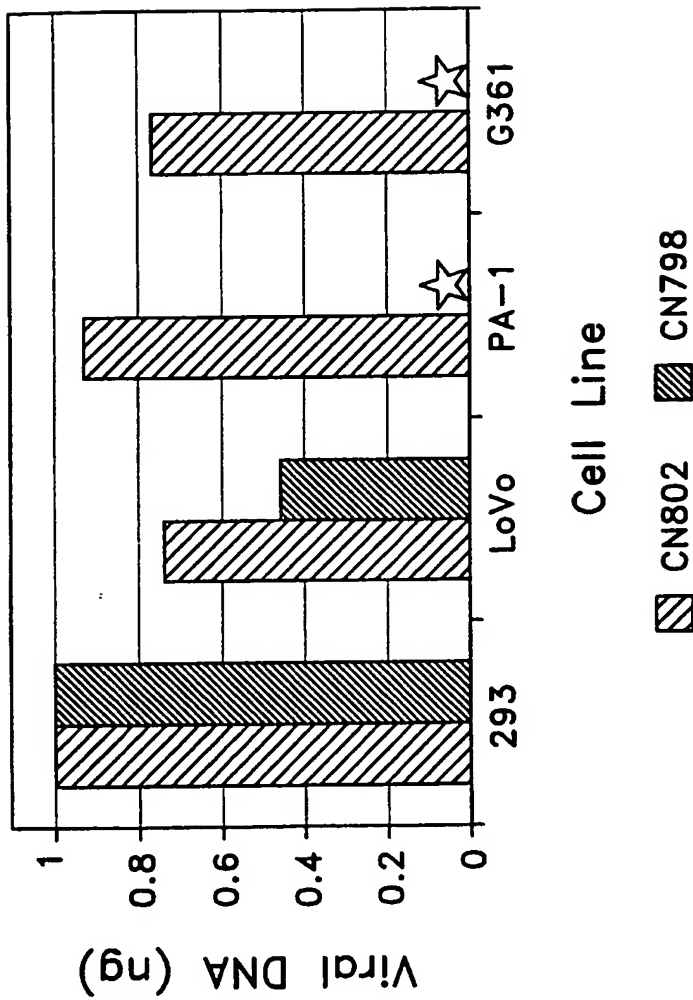(CEA-TRE)
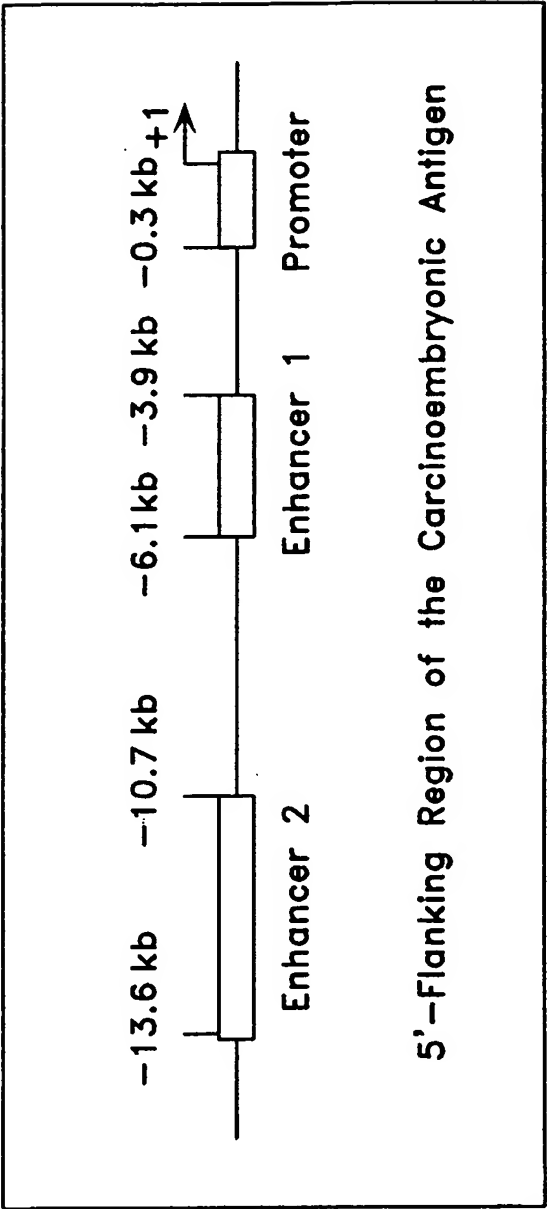
FIGURE 15

FIGURE 16

FIGURE 17

FIGURE 18

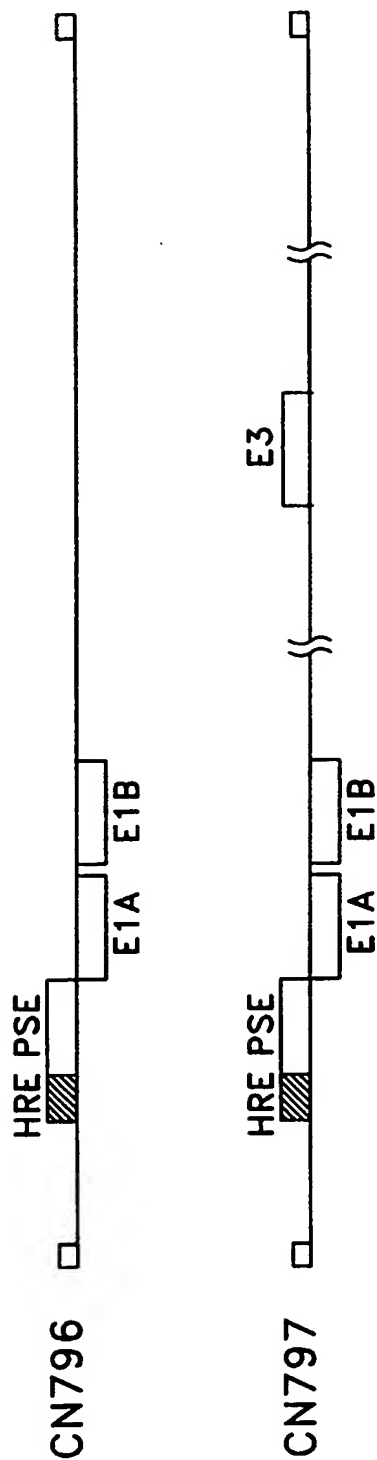FIGURE 19

FIGURE 20

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FIGURE 21

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FIGURE 22A

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SUBSTITUTE SHEET (RULE 26)

FIGURE 22B

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aagacacaca gatacgtgac agaaccatgg agaattgcct cccaacgctg ttcagccaga 1920

gccttccacc cttgtctgca ggacagtctc aacgttccac cattaaatac ttcttctatc 1980

acatcctgct tctttatgcc taaccaaggt tctaggtccc gatcgactgt gtctggcagc 2040

actccactgc caaacccaga ataaggcagc gctcaggatc ccgaaggggc atggctgggg 2100

atcagaactt ctgggtttga gtgaggagtg ggtccaccct cttgaatttc aaaggaggaa 2160

gaggctggat gtgaaggtac tgggggaggg aaagtgtcag ttccgaactc ttaggtcaat 2220

gagggaggag actggtaagg tcccagctcc cgaggtactg atgtgggaat ggcctaagaa 2280

tctcatatcc tcaggaagaa ggtgctggaa tcctgagggg tagagttctg ggtatatttg 2340

tggcttaagg ctctttggcc cctgaaggca gaggctggaa ccattaggtc cagggtttgg 2400

ggtgatagta atggatctc ttgattcctc aagagtctga ggatcgaggg ttgcccattc 2460

ttccatcttg ccacctaatc cttactccac ttgagggtat caccagccct tctagctcca 2520

tgaaggtccc ctgggcaagc acaatctgag catgaaagat gccccagagg ccttgggtgt 2580

catccactca tcatccagca tcacactctg agggtgtggc cagcaccatg acgtcatgtt 2640

gctgtgacta tccctgcagc gtgcctctcc agccacctgc caaccgtaga gctgcccatc 2700

ctcctctggt gggagtggcc tgcatggtgc caggctgagg cctagtgtca gacagggagc 2760

ctggaatcat agggatccag gactcaaaag tgctagagaa tggccatatg tcaccatcca 2820

tgaaatctca agggcttctg ggtggagggc acaggacct gaacttatgg tttcccaagt 2880

ctattgctct cccaagtgag tctccagat acgaggcact gtgccagcat cagccttatc 2940

tccaccacat cttgtaaaag gactacccag ggccctgatg aacaccatgg tgtgtacagg 3000

agtaggggt ggaggcacgg actcctgtga ggtcacagcc aagggagcat catcatgggt 3060

ggggaggagg caatggacag gcttgagaac ggggatgtgg ttgtatttgg ttttctttgg 3120

ttagataaag tgctgggtat aggattgaga gtggagtatg aagaccagtt aggatggagg 3180

atcagattgg acttgggtta gataaagtgc tgggtatagg attgagagtg gagtatgaag 3240

accagttagg atggaggatc agattggagt tgggttagag atggggtaaa attgtgctcc 3300

ggatgagttt gcgattgaca ctgtcgaggt ggtttgggat ggcatggctt tgggatggaa 3360
```

FIGURE 22C

```
atagatttgt tttgatgttg gctcagacat ccttggggat tgaactgggg atgaagctgg 3420

gtttgatttt ggaggtagaa gacgtggaag tagctgtcag atttgacagt ggccatgagt 3480

tttgtttgat ggggaatcaa acaatggggg aagacataag ggttggcttg ttaggttaag 3540

ttgcgttggg ttgatggggt cggggctgtg tataatgcag ttggattggt ttgtattaaa 3600

ttgggttggg tcaggttttg gttgaggatg agttgaggat atgcttgggg acaccggatc 3660

catgaggttc tcactggagt ggagacaaac ttcctttcca ggatgaatcc agggaagcct 3720

taattcacgt gtaggggagg tcaggccact ggctaagtat atccttccac tccagctcta 3780

agatggtctt aaattgtgat tatctatatc cacttctgtc tccctcactg tgcttggagt 3840

ttacctgatc actcaactag aaacagggga agattttatc aaattctttt ttttttttt 3900

tttttttga dacagagtct cactctgttg cccaggctgg agtgcagtgg cgcagtctcg 3960

gctcactgca acctctgcct cccaggttca agtgattctc ctgcctcagc ctcctgagtt 4020

gctgggatta caggcatgca gcaccatgcc cagctaattt ttgtattttt agtagagatg 4080

gggtttcacc aatgtttgcc aggctggcct cgaactcctg acctggtgat ccacctgcct 4140

cagcctccca aagtgctggg attacaggcg tcagccaccg cgcccagcca cttttgtcaa 4200

attcttgaga cacagctcgg gctggatcaa gtgagctact ctggttttat tgaacagctg 4260

aaataaccaa ctttttggaa attgatgaaa tcttacggag ttaacagtgg aggtaccagg 4320

gctcttaaga gttcccgatt ctcttctgag actacaaatt gtgattttgc atgccacctt 4380

aatcttttt ttttttttt taaatcgagg tttcagtctc attctatttc ccaggctgga 4440

gttcaatagc gtgatcacag ctcactgtag ccttgaactc ctggccttaa gagattctcc 4500

tgcttcggtc tcccaatagc taagactaca gtagtccacc accatatcca gataattttt 4560

aaatttttg ggggccgggc cacagtggct cacgcctgta atcccaacac catgggaggc 4620

tgagatgggt ggatcacgag gtcaggagtt tgagaccagc ctgaccaaca tggtgaaact 4680

ctgtctctac taaaaaaaaa aaaatagaa aaattagccg ggcgtggtgg cacacggcac 4740

ctgtaatccc agctactgag gaggctgagg caggagaatc acttgaaccc agaaggcaga 4800

ggttgcaatg agccgagatt gcgccactgc actccagcct gggtgacaga gtgagactct 4860

gtctcaaaaa aaaaaaattt ttttttttt tttgtagaga tggatcttgc tttgtttctc 4920

tggttggcct tgaactcctg gcttcaagtg atcctcctac cttggcctcg gaaagtgttg 4980

ggattacagg cgtgagccac catgactgac ctgtcgttaa tcttgaggta cataaacctg 5040

gctcctaaag gctaaaggct aaatatttgt tggagaaggg gcattggatt ttgcatgagg 5100
```

FIGURE 22D

```
atgattctga cctgggaggg caggtcagca ggcatctctg ttgcacagat agagtgtaca 5160

ggtctggaga acaaggagtg gggggttatt ggaattccac attgtttgct gcacgttgga 5220

ttttgaaatg ctagggaact ttgggagact catatttctg ggctagagga tctgtggacc 5280

acaagatctt tttatgatga cagtagcaat gtatctgtgg agctggattc tgggttggga 5340

gtgcaaggaa aagaatgtac taaatgccaa gacatctatt tcaggagcat gaggaataaa 5400

agttctagtt tctggtctca gagtggtgca gggatcaggg agtctcacaa tctcctgagt 5460

gctggtgtct tagggcacac tgggtcttgg agtgcaaagg atctaggcac gtgaggcttt 5520

gtatgaagaa tcggggatcg tacccacccc ctgtttctgt ttcatcctgg gcatgtctcc 5580

tctgcctttg tcccctagat gaagtctcca tgagctacaa gggcctggtg catccagggt 5640

gatctagtaa ttgcagaaca gcaagtgcta gctctccctc cccttccaca gctctgggtg 5700

tgggaggggg ttgtccagcc tccagcagca tggggagggc cttggtcagc ctctgggtgc 5760

cagcagggca ggggcggagt cctggggaat gaaggtttta tagggctcct gggggaggct 5820
                                                                   5836
ccccagcccc aagctt
```

FIGURE 23A

```
aagctttta gtgctttaga cagtgagctg gtctgtctaa cccaagtgac ctggctcca      60

tactcagccc cagaagtgaa gggtgaagct gggtggagcc aaaccaggca agcctaccct    120

cagggctccc agtggcctga gaaccattgg acccaggacc cattacttct agggtaagga    180

aggtacaaac accagatcca accatggtct gggggacag ctgtcaaatg cctaaaata     240

tacctgggag aggagcaggc aaactatcac tgccccaggt tctctgaaca gaaacagagg    300

ggcaacccaa agtccaaatc caggtgagca ggtgcaccaa atgcccagag atatgacgag    360

gcaagaagtg aaggaaccac ccctgcatca aatgttttgc atgggaagga gaaggggggtt   420

gctcatgttc ccaatccagg agaatgcatt tgggatctgc cttcttctca ctccttggtt    480

agcaagacta agcaaccagg actctggatt tggggaaaga cgtttatttg tggaggccag    540

tgatgacaat cccacgaggg cctaggtgaa gagggcagga aggctcgaga cactggggac    600 ·

tgagtgaaaa ccacacccat gatctgcacc acccatggat gctccttcat tgctcacctt    660

tctgttgata tcagatggcc ccattttctg taccttcaca gaaggacaca ggctagggtc    720

tgtgcatggc cttcatcccc ggggccatgt gaggacagca ggtgggaaag atcatgggtc    780

ctcctgggtc ctgcagggcc agaacattca tcacccatac tgacctccta gatgggaatg    840

gcttccctgg ggctgggcca acggggcctg ggcaggggag aaaggacgtc aggggacagg    900

gaggaagggt catcgagacc cagcctggaa ggttcttgtc tctgaccatc caggatttac    960

ttccctgcat ctacctttgg tcattttccc tcagcaatga ccagctctgc ttcctgatct   1020

cagcctccca ccctggacac agcaccccag tccctggccc ggctgcatcc acccaatacc   1080

ctgataaccc aggacccatt acttctaggg taaggagggt ccaggagaca gaagctgagg   1140

aaaggtctga agaagtcaca tctgtcctgg ccagagggga aaaaccatca gatgctgaac   1200

caggagaatg ttgacccagg aaagggaccg aggacccaag aaaggagtca gaccaccagg   1260

gtttgcctga gaggaaggat caaggccccg agggaaagca gggctggctg catgtgcagg   1320

acactggtgg ggcatatgtg tcttagattc tccctgaatt cagtgtccct gccatggcca   1380

gactctctac tcaggcctgg acatgctgaa ataggacaat ggccttgtcc tctctcccca   1440

ccatttggca agagacataa aggacattcc aggacatgcc ttcctgggag gtccaggttc   1500

tctgtctcac acctcaggga ctgtagttac tgcatcagcc atggtaggtg ctgatctcac   1560

ccagcctgtc caggcccttc cactctccac tttgtgacca tgtccaggac cacccctcag   1620

atcctgagcc tgcaaatacc cccttgctgg gtgggtggat tcagtaaaca gtgagctcct   1680
```

FIGURE 23B

```
atccagcccc gagagccacc tctgtcacct tcctgctggg catcatccca ccttcacaag    1740

cactaaagag catggggaga cctggctagc tgggtttctg catcacaaag aaaataatcc    1800

cccaggttcg gattcccagg gctctgtatg tggagctgac agacctgagg ccaggagata    1860

gcacaggtca gccctaggga gggtgggtca tccacccagg ggacaggggt gcaccagcct    1920

tgctactgaa agggcctccc caggacagcg ccatcagccc tgcctgagag ctttgctaaa    1980

cagcagtcag aggaggccat ggcagtggct gagctcctgc tccaggcccc aacagaccag    2040

accaacagca caatgcagtc cttccccaac gtcacaggtc accaaaggga aactgaggtg    2100

ctacctaacc ttagagccat caggggagat aacagcccaa tttcccaaac aggccagttt    2160

caatcccatg acaatgacct ctctgctctc attcttccca aaataggacg ctgattctcc    2220

cccaccatgg atttctccct tgtcccggga gccttttctg ccccctatga tctgggcact    2280

cctgacacac acctcctctc tggtgacata tcagggtccc tcactgtcaa gcagtccaga    2340

aagacagaa ccttggacag cgcccatctc agcttcaccc ttcctccttc acagggttca    2400

gggcaaagaa taaatggcag aggccagtga gcccagagat ggtgacaggc agtgacccag    2460

gggcagatgc ctggagcagg agctggcggg gccacaggga gaaggtcatg caggaaggga    2520

aacccagaaa tgggcaggaa aggaggacac aggctctgtg gggctgcagc ccagggttgg    2580

actatgagtg tgaagccatc tcagcaagta aggccaggtc ccatgaacaa gagtgggagc    2640

acgtggcttc ctgctctgta tatggggtgg gggattccat gccccataga accagatggc    2700

cggggttcag atggagaagg agcaggacag gggatcccca ggataggagg accccagtgt    2760

ccccacccag gcaggtgact gatgaatggg catgcagggt cctcctgggc tgggctctcc    2820

ctttgtccct caggattcct tgaaggaaca tccggaagcc gaccacatct acctggtggg    2880

ttctggggag tccatgtaaa gccaggagct tgtgttgcta ggaggggtca tggcatgtgc    2940

tggcggcacc aaagagagaa acctgagggc aggcaggacc tggtctgagg aggcatggga    3000

gcccagatgg ggagatggat gtcaggaaag gctgccccat cagggagggt gatagcaatg    3060

gggggtctgt gggagtgggc acgtgggatt ccctgggctc tgccaagttc cctcccatag    3120

tcacaacctg gggacactgc ccatgaaggg gcgcctttgc ccagccagat gctgctggtt    3180

ctgcccatcc actaccctct ctgctccagc cactctgggt cttttctccag atgccctgga    3240

cagccctggc ctgggcctgt cccctgagag gtgttgggag aagctgagtc tctggggaca    3300

ctctcatcag agtctgaaag gcacatcagg aaacatccct ggtctccagg actaggcaat    3360
```

FIGURE 23C

```
gaggaaaggg ccccagctcc tcccttttcc actgagaggg tcgaccctgg gtggccacag   3420

tgacttctgc gtctgtccca gtcaccctga aaccacaaca aaaccccagc cccagaccct   3480

gcaggtacaa tacatgtggg gacagtctgt acccagggga agccagttct ctcttcctag   3540

gagaccgggc ctcagggctg tgcccggcgc aggcgggggc agcacgtgcc tgtccttgag   3600

aactcgggac cttaagggtc tctgctctgt gaggcacagc aaggatcctt ctgtccagag   3660

atgaaagcag ctcctgcccc tcctctgacc tcttcctcct tcccaaatct caaccaacaa   3720

ataggtgttt caaatctcat catcaaatct tcatccatcc acatgagaaa gcttaaaacc   3780

caatggattg acaacatcaa gagttggaac aagtggacat ggagatgtta cttgtggaaa   3840

tttagatgtg ttcagctatc gggcaggaga atctgtgtca aattccagca tggttcagaa   3900

gaatcaaaaa gtgtcacagt ccaaatgtgc aacagtgcag gggataaaac tgtggtgcat   3960

tcaaactgag ggatattttg gaacatgaga aaggaaggga ttgctgctgc acagaacatg   4020

gatgatctca cacatagagt tgaaagaaag gagtcaatcg cagaatagaa aatgatcact   4080

aattccacct ctataaagtt tccaagagga aaacccaatt ctgctgctag agatcagaat   4140

ggaggtgacc tgtgccttgc aatggctgtg agggtcacgg gagtgtcact tagtgcaggc   4200

aatgtgccgt atcttaatct gggcagggct ttcatgagca cataggaatg cagacattac   4260

tgctgtgttc attttacttc accggaaaag aagaataaaa tcagccgggc gcggtggctc   4320

acgcctgtaa tcccagcact ttagaaggct gaggtgggca gattacttga ggtcaggagt   4380

tcaagaccac cctggccaat atggtgaaac cccggctcta ctaaaaatac aaaaattagc   4440

tgggcatggt ggtgcgcgcc tgtaatccca gctactcggg aggctgaggc tggacaattg   4500

cttggaccca ggaagcagag gttgcagtga gccaagattg tgccactgca ctccagcttg   4560

ggcaacagag ccagactctg taaaaaaaaa aaaaaaaaaa aaaaaagaa agaaagaaaa   4620

agaaaagaaa gtataaaatc tctttgggtt aacaaaaaaa gatccacaaa acaaacacca   4680

gctcttatca aacttacaca actctgccag agaacaggaa acacaaatac tcattaactc   4740

acttttgtgg caataaaacc ttcatgtcaa aaggagacca ggacacaatg aggaagtaaa   4800

actgcaggcc ctacttgggt gcagagaggg aaaatccaca aataaaacat taccagaagg   4860

agctaagatt tactgcattg agttcattcc ccaggtatgc aaggtgattt taacacctga   4920

aaatcaatca ttgcctttac tacatagaca gattagctag aaaaaaatta caactagcag   4980

aacagaagca atttggcctt cctaaaattc cacatcatat catcatgatg gagacagtgc   5040

agacgccaat gacaataaaa agagggacct ccgtcacccg gtaaacatgt ccacacagct   5100
```

SUBSTITUTE SHEET (RULE 26)

FIGURE 23D

```
ccagcaagca—eccgtcttcc cagtgaatca ctctaacctc ccctttaatc agccccaggc    5160

aaggctgcct gcgatggcca cacaggctcc aacccgtggg cctcaacctc ccgcagaggc    5220

tctcctttgg ccaccccatg gggagagcat gaggacaggg cagagccctc tgatgcccac    5280

acatggcagg agctgacgcc agagccatgg gggctggaga gcagagctgc tggggtcaga    5340

gcttcctgag gacacccagg cctaagggaa ggcagctccc tggatggggg caaccaggct    5400

ccgggctcca acctcagagc ccgcatggga ggagccagca ctctaggcct ttcctagggt    5460

gactctgagg ggaccctgac acgacaggat cgctgaatgc acccgagatg aaggggccac    5520

cacgggaccc tgctctcgtg gcagatcagg agagagtggg acaccatgcc aggcccccat    5580

ggcatggctg cgactgaccc aggccactcc cctgcatgca tcagcctcgg taagtcacat    5640

gaccaagccc aggaccaatg tggaaggaag gaaacagcat cccctttagt gatggaaccc    5700

aaggtcagtg caaagagagg ccatgagcag ttaggaaggg tggtccaacc tacagcacaa    5760

accatcatct atcataagta gaagccctgc tccatgaccc ctgcatttaa ataaacgttt    5820

gttaaatgag tcaaattccc tcaccatgag agctcacctg tgtgtaggcc catcacacac    5880

acaaacacac acacacacac acacacacac acacacacac acagggaaag tgcaggatcc    5940

tggacagcac caggcaggct tcacaggcag agcaaacagc gtgaatgacc catgcagtgc    6000

cctgggcccc atcagctcag agaccctgtg agggctgaga tggggctagg caggggagag    6060

acttagagag ggtggggcct ccagggaggg ggctgcaggg agctgggtac tgccctccag    6120

ggagggggct gcagggagct gggtactgcc ctccagggag ggggctgcag ggagctgggt    6180

actgccctcc agggaggggg ctgcagggag ctgggtactg ccctccaggg aggggctgc    6240

agggagctgg gtactgccct ccagggaggc aggagcactg ttcccaacag agagcacatc    6300

ttcctgcagc agctgcacag acacaggagc ccccatgact gccctgggcc agggtgtgga    6360

ttccaaattt cgtgccccat tgggtgggac ggaggttgac cgtgacatcc aagggcatc    6420

tgtgattcca aacttaaact actgtgccta caaaatagga aataacccta cttttctac    6480

tatctcaaat tccctaagca caagctagca ccctttaaat caggaagttc agtcactcct    6540

ggggtcctcc catgcccca gtctgacttg caggtgcaca gggtggctga catctgtcct    6600

tgctcctcct cttggctcaa ctgccgcccc tcctgggggt gactgatggt caggacaagg    6660

gatcctagag ctggccccat gattgacagg aaggcaggac ttggcctcca ttctgaagac    6720

taggggtgtc aagagagctg ggcatcccac agagctgcac aagatgacgc ggacagaggg    6780
```

FIGURE 23E

```
tgacacaggg ctcagggctt cagacgggtc gggaggctca gctgagagtt caggcacaga   6840

cctgaggagc ctcagtggga aaagaagcac tgaagtggga agttctggaa tgttctggac   6900

aagcctgagt gctctaagga aatgctccca ccccgatgta gcctgcagca ctggacggtc   6960

tgtgtacctc cccgctgccc atcctctcac agcccccgcc tctagggaca caactcctgc   7020

cctaacatgc atctttcctg tctcattcca cacaaaaggg cctctggggt ccctgttctg   7080

cattgcaagg agtggaggtc acgttcccac agaccaccca gcaacaggt cctatggagg   7140

tgcggtcagg aggatcacac gtccccccat gcccagggga ctgactctgg gggtgatgga   7200

ttggcctgga ggccactggt cccctctgtc cctgagggga atctgcaccc tggaggctgc   7260

cacatccctc ctgattcttt cagctgaggg cccttcttga atcccaggg aggactcaac   7320

ccccactggg aaaggcccag tgtggacggt tccacagcag cccagctaag gcccttggac   7380

acagatcctg agtgagagaa cctttaggga cacaggtgca cggccatgtc cccagtgccc   7440

acacagagca ggggcatctg gaccctgagt gtgtagctcc cgcgactgaa cccagccctt   7500

ccccaatgac gtgacccctg gggtggctcc aggtctccag tccatgccac caaaatctcc   7560

agattgaggg tcctcccttg agtccctgat gcctgtccag gagctgcccc ctgagcaaat   7620

ctagagtgca gagggctggg attgtggcag taaaagcagc cacatttgtc tcaggaagga   7680

aagggaggac atgagctcca ggaagggcga tggcgtcctc tagtgggcgc ctcctgttaa   7740

tgagcaaaaa ggggccagga gagttgagag atcagggctg gccttggact aaggctcaga   7800

tggagaggac tgaggtgcaa agaggggggct gaagtagggg agtggtcggg agagatggga   7860

ggagcaggta aggggaagcc ccagggaggc cgggggaggg tacagcagag ctctccactc   7920

ctcagcattg acatttgggg tggtcgtgct agtggggttc tgtaagttgt agggtgttca   7980

gcaccatctg gggactctac ccactaaatg ccagcaggac tccctcccca agctctaaca   8040

accaacaatg tctccagact ttccaaatgt cccctggaga gcaaaattgc ttctggcaga   8100

atcactgatc tacgtcagtc tctaaaagtg actcatcagc gaaatccttc acctcttggg   8160

agaagaatca caagtgtgag aggggtagaa actgcagact tcaaaatctt tccaaaagag   8220

ttttacttaa tcagcagttt gatgtccag gagaagatac atttagagtg tttagagttg   8280

atgccacatg gctgcctgta cctcacagca ggagcagagt gggttttcca agggcctgta   8340

accacaactg gaatgacact cactgggtta cattacaaag tggaatgtgg ggaattctgt   8400

agactttggg aagggaaatg tatgacgtga gcccacagcc taaggcagtg gacagtccac   8460

tttgaggctc tcaccatcta ggagacatct cagccatgaa catagccaca tctgtcatta   8520
```

FIGURE 23F

```
gaaaacatgt-tttattaaga ggaaaaatct aggctagaag tgcttतatgc tctttttttct    8580

ctttatgttc aaattcatat acttttagat cattccttaa agaacaatct atcccctaa     8640

gtaaatgtta tcactgactg gatagtgttg gtgtctcact cccaacccct gtgtggtgac    8700

agtgccctgc ttccccagcc ctgggccctc tctgattcct gagacttttg ggtgctcctt    8760

cattaggagg aagagaggaa gggtgttttt aatattctca ccattcaccc atccacctct    8820

tagacactgg gaagaatcag ttgcccactc ttggatttga tcctcgaatt aatgacctct    8880

atttctgtcc cttgtccatt tcaacaatgt gacaggccta agaggtgcct tctccatgtg    8940

atttttgagg agaaggttct caagataagt tttctcacac ctctttgaat tacctccacc    9000

tgtgtcccca tcaccattac cagcagcatt tggacccttt ttctgttagt cagatgcttt    9060

ccacctcttg agggtgtata ctgtatgctc tctacacagg aatatgcaga ggaaatagaa    9120

aaagggaaat cgcattacta ttcagagaga agaagacctt tatgtcaatg aatgagagtc    9180

taaaatccta agagagccca tataaaatta ttaccagtgc taaaactaca aaagttacac    9240

taacagtaaa ctagaataat aaaacatgca tcacagttgc tggtaaagct aaatcagata    9300

ttttttttctt agaaaaagca ttccatgtgt gttgcagtga tgacaggagt gcccttcagt    9360

caatatgctg cctgtaattt ttgttccctg gcagaatgta ttgtcttttc tccctttaaa    9420

tcttaaatgc aaaactaaag gcagctcctg ggccccctcc ccaaagtcag ctgcctgcaa    9480

ccagccccac gaagagcaga ggcctgagct tccctggtca aaatacgggg ctagggagct    9540

taaccttgct cgataaagct gtgttcccag aatgtcgctc ctgttcccag gggcaccagc    9600

ctggagggtg gtgagcctca ctggtggcct gatgcttacc ttgtgccctc acaccagtgg    9660

tcactggaac cttgaacact tggctgtcgc ccggatctgc agatgtcaag aacttctgga    9720

agtcaaatta ctgcccactt ctccagggca gatacctgtg aacatccaaa accatgccac    9780

agaaccctgc ctggggtcta caacacatat ggactgtgag caccaagtcc agccctgaat    9840

ctgtgaccac ctgccaagat gcccctaact gggatccacc aatcactgca catggcaggc    9900

agcgaggctt ggaggtgctt cgccacaagg cagcccaat ttgctgggag tttcttggca    9960

cctggtagtg gtgaggagcc ttgggaccct caggattact ccccttaagc atagtgggga   10020

cccttctgca tccccagcag gtgccccgct cttcagagcc tctctctctg aggtttaccc   10080

agacccctgc accaatgaga ccatgctgaa gcctcagaga gagagatgga gctttgacca   10140

ggagccgctc ttccttgagg gccagggcag ggaaagcagg aggcagcacc aggagtggga   10200
```

FIGURE 23G

```
acaccagtgt ctaagcccct gatgagaaca gggtggtctc tcccatatgc ccataccagg   10260

cctgtgaaca gaatcctcct tctgcagtga caatgtctga gaggacgaca tgtttcccag   10320

cctaacgtgc agccatgccc atctacccac tgcctactgc aggacagcac caacccagga   10380

gctgggaagc tgggagaaga catggaatac ccatggcttc tcaccttcct ccagtccagt   10440

gggcaccatt tatgcctagg acacccacct gccggcccca ggctcttaag agttaggtca   10500

cctaggtgcc tctgggaggc cgaggcagga gaattgcttg aacccgggag gcagaggttg   10560

cagtgagccg agatcacacc actgcactcc agcctgggtg acagaatgag actctgtctc   10620

aaaaaaaaag agaaagatag catcagtggc taccaagggc taggggcagg ggaaggtgga   10680

gagttaatga ttaatagtat gaagtttcta tgtgagatga tgaaaatgtt ctggaaaaaa   10740

aaatatagtg gtgaggatgt agaatattgt gaatataatt aacggcattt aattgtacac   10800

ttaacatgat taatgtggca tattttatct tatgtatttg actacatcca agaaacactg   10860

ggagagggaa agcccaccat gtaaaataca cccaccctaa tcagatagtc ctcattgtac   10920

ccaggtacag gcccctcatg acctgcacag gaataactaa ggatttaagg acatgaggct   10980

tcccagccaa ctgcaggtgc acaacataaa tgtatctgca aacagactga gagtaaagct   11040

gggggcacaa acctcagcac tgccaggaca cacaccttc tcgtggattc tgactttatc   11100

tgacccggcc cactgtccag atcttgttgt gggattggga caagggaggt cataaagcct   11160

gtccccaggg cactctgtgt gagcacacga gacctcccca cccccccacc gttaggtctc   11220

cacacataga tctgaccatt aggcattgtg aggaggactc tagcgcgggc tcagggatca   11280

caccagagaa tcaggtacag agaggaagac ggggctcgag gagctgatgg atgacacaga   11340

gcagggttcc tgcagtccac aggtccagct caccctggtg taggtgcccc atcccctga   11400

tccaggcatc cctgacacag ctccctcccg gagcctcctc ccaggtgaca catcagggtc   11460

cctcactcaa gctgtccaga gagggcagca ccttggacag cgcccacccc acttcactct   11520

tcctccctca cagggctcag ggctcagggc tcaagtctca gaacaaatgg cagaggccag   11580

tgagcccaga gatggtgaca gggcaatgat ccaggggcag ctgcctgaaa cgggagcagg   11640

tgaagccaca gatgggagaa gatggttcag gaagaaaaat ccaggaatgg gcaggagagg   11700

agaggaggac acaggctctg tggggctgca gcccaggatg ggactaagtg tgaagacatc   11760

tcagcaggtg aggccaggtc ccatgaacag agaagcagct cccacctccc ctgatgcacg   11820

gacacacaga gtgtgtggtg ctgtgccccc agagtcgggc tctcctgttc tggtccccag   11880

ggagtgagaa gtgaggttga cttgtccctg ctcctctctg ctaccccaac attcaccttc   11940
```

FIGURE 23H

```
tcctcatgcc-cctctctctc aaatatgatt tggatctatg tccccgccca aatctcatgt   12000

caaattgtaa accccaatgt tggaggtggg gccttgtgag aagtgattgg ataatgcggg   12060

tggattttct gctttgatgc tgtttctgtg atagagatct cacatgatct ggttgtttaa   12120

aagtgtgtag cacctctccc ctctctctct ctctctctta ctcatgctct gccatgtaag   12180

acgttcctgt ttccccttca ccgtccagaa tgattgtaag ttttctgagg cctccccagg   12240

agcagaagcc actatgcttc ctgtacaact gcagaatgat gagcgaatta aacctctttt   12300

ctttataaat tacccagtct caggtatttc tttatagcaa tgcgaggaca gactaataca   12360

atcttctact cccagatccc cgcacacgct tagccccaga catcactgcc cctgggagca   12420

tgcacagcgc agcctcctgc cgacaaaagc aaagtcacaa aaggtgacaa aaatctgcat   12480

ttggggacat ctgattgtga aagagggagg acagtacact tgtagccaca gagactgggg   12540

ctcaccgagc tgaaacctgg tagcactttg gcataacatg tgcatgaccc gtgttcaatg   12600

tctacagatc agtgttgagt aaaacagcct ggtctggggc cgctgctgtc cccacttccc   12660

tcctgtccac cagagggcgg cagagttcct cccaccctgg agcctcccca ggggctgctg   12720

acctccctca gccgggccca cagcccagca gggtccaccc tcacccgggt cacctcggcc   12780

cacgtcctcc tcgccctccg agctcctcac acggactctg tcagctcctc cctgcagcct   12840

atcggccgcc cacctgaggc ttgtcggccg cccacttgag gcctgtcggc tgccctctgc   12900

aggcagctcc tgtcccctac accccctcct tccccgggct cagctgaaag ggcgtctccc   12960

agggcagctc cctgtgatct ccaggacagc tcagtctctc acaggctccg acgcccccta   13020

tgctgtcacc tcacagccct gtcattacca ttaactcctc agtcccatga agttcactga   13080

gcgcctgtct cccggttaca ggaaaactct gtgacaggga ccacgtctgt cctgctctct   13140

gtggaatccc agggcccagc ccagtgcctg acacggaaca gatgctccat aaatactggt   13200

taaatgtgtg ggagatctct aaaaagaagc atatcacctc cgtgtggccc ccagcagtca   13260

gagtctgttc catgtggaca caggggcact ggcaccagca tgggaggagg ccagcaagtg   13320

cccgcggctg ccccaggaat gaggcctcaa cccccagagc ttcagaaggg aggacagagg   13380

cctgcaggga atagatcctc cggcctgacc ctgcagccta atccagagtt cagggtcagc   13440

tcacaccacg tcgaccctgg tcagcatccc tagggcagtt ccagacaagg ccggaggtct   13500

cctcttgccc tccagggggt gacattgcac acagacatca ctcaggaaac ggattcccct   13560

ggacacgaac ctggctttgc taaggaagtg gaggtggagc ctggtttcca tcccttgctc   13620
```

FIGURE 23I

```
caacagaccc ttctgatctc tcccacatac ctgctctgtt cctttctcgg tcctatgagg   13680

accctgttct gccaggggtc cctgtgcaac tccagactcc ctcctggtac caccatgggg   13740

aaggtggggt gatcacagga cagtcagcct cgcagagaca cagaccaccc aggactgtca   13800

gggagaacat ggacaggccc tgagccgcag ctcagccaac agacacgcag agggagggtc   13860

cccctggagc cttccccaag gacagcagag cccagagtca cccacctccc tccaccacag   13920

tcctctcttt ccaggacaca caagacacct ccccctccac atgcaggatc tggggactcc   13980

tgagacctct gggcctgggt ctccatccct gggtcagtgg cggggttggt ggtactggag   14040

acagagggct ggtccctccc cagccaccac ccagtgagcc ttttctagc ccccagagcc   14100

acctctgtca ccttcctgtt gggcatcatc ccaccttccc agagccctgg agagcatggg   14160

gagacccggg accctgctgg gtttctctgt cacaaaggaa aataatcccc ctggtgtgac   14220

agacccaagg acagaacaca gcagaggtca gcactgggga agacaggttg tcctcccagg   14280

ggatgggggt ccatccacct tgccgaaaag atttgtctga ggaactgaaa atagaaggga   14340

aaaaagagga gggacaaaag aggcagaaat gagaggggag cggacagacg acacctgaat   14400

aaacaccaca cccatgaccc acgtgatgct gagaagtact cctgccctag gaagagactc   14460
         ↱
aggccagagg gaggaaggac agcagaccag acagtcacag cagccttgac aaaacgttcc   14520

tggaactcaa gctcttctcc acagaggagg acagagcaga cagcagagac catggagtct   14580

ccctcggccc ctccccacag atggtgcatc ccctggcaga ggctcctgct cacaggtgaa   14640

gggaggacaa cctgggagag ggtgggagga gggagctggg gtctcctgcg taggacaggg   14700

ctgtgagacg gacagagggc tcctgttgga gcctgaatag cgaagaggac atcagagagg   14760

gacaggagtc acaccagaaa aatcaaattg aactggaatt gcaaagggc aggaaaacct   14820

caagagttct attttcctag ttaattgtca ctggccacta cgttttaaa aatcataata   14880

actgcatcag atgacacttt aaataaaaac ataaccaggg catgaaacac tgtcctcatc   14940

cgcctaccgc ggacattgga aaataagccc caggctgtgg agggccctgg gaaccctcat   15000

gaactcatcc acaggaatct gcagcctgtc ccaggcactg gggtgcaacc aagatc       15056
```

FIGURE 24A

```
gaattcagaa ataggggaag gttgaggaag gacactgaac tcaaacggga tacagtgatt 60

ggtttatttg tcttctcttc acaacattgg tgctggagga attcccaccc tgaggttatg 120

aagatgtctg aacacccaac acatagcact ggagatatga gctcgacaag agtttctcag 180

ccacagagat tcacagccta gggcaggagg acactgtacg ccaggcagaa tgacatggga 240

attgcgctca cgattggctt gaagaagcaa ggactgtggg aggtgcgctt tgtagtaaca 300

agagggcagg gtgaactctg attcccatgg gggaatgtga tggtcctgtt acaaattttt 360

caagctggca gggaataaaa cccattacgg tgaggacctg tggagggcgg ctgccccaac 420

tgataaagga aatagccagg tgggggcctt tcccattgta gggggggacat atctggcaat 480

agaagccttt gagacccttt agggtacaag tactgaggca gcaaataaaa tgaaatctta 540

tttttcaact ttatactgca tgggtgtgaa gatatatttg tttctgtaca gggggtgagg 600

gaaaggaggg gaggaggaaa gttcctgcag gtctggtttg gtcttgtgat ccaggggggtc 660

ttggaactat ttaaattaaa ttaaattaaa acaagcgact gttttaaatt aaattaaatt 720

aaattaaatt ttactttatt ttatcttaag ttctgggcta catgtgcagg acgtgcagct 780

ttgttacata ggtaaacgtg tgccatggtg gtttgctgta cctatcaacc catcacctag 840

gtattaagcc cagcatgcat tagctgtttt tcctgacgct ctccctctcc ctgactccca 900

caacaggccc cagtgtgtgt tgttcccctc cctgtgtcca tgtgttctca ttgttcagct 960

cccacttata agtgagaaca tgtggtgttt ggttttctgt ttctgtgtta gtttgctgag 1020

gataatggct tccacctcca tccatgttcc tgcaaaggac gtgatcttat tcttttttat 1080

ggttgcatag aaattgtttt tacaaatcca attgatattg tatttaatta caagttaatc 1140

taattagcat actagaagag attacagaag atattaggta cattgaatga ggaaatatat 1200

aaaataggac gaaggtgaaa tattaggtag gaaaagtata atagttgaaa gaagtaaaaa 1260

aaaatatgca tgagtagcag aatgtaaaag aggtgaagaa cgtaatagtg actttttaga 1320

ccagattgaa ggacagagac agaaaaattt taaggaattg ctaaaccatg tgagtgttag 1380

aagtacagtc aataacatta aagcctcagg aggagaaaag aataggaaag gaggaaatat 1440

gtgaataaat agtagagaca tgtttgatgg attttaaaat atttgaaaga cctcacatca 1500

aaggattcat accgtgccat tgaagaggaa gatggaaaag ccaagaagcc agatgaaagt 1560

tagaaatatt attggcaaag cttaaatgtt aaaagtccta gagagaaagg atggcagaaa 1620

tattggcggg aaagaatgca gaacctagaa tataaattca tcccaacagt ttggtagtgt 1680
```

FIGURE 24B

```
gcagctgtag cctttttctag ataatacact attgtcatac atcgcttaag cgagtgtaaa 1740

atggtctcct cactttattt atttatatat ttatttagtt ttgagatgga gcctcgctct 1800

gtctcctagg ctggagtgca atagtgcgat accactcact gcaacctctg cctcctctgt 1860

tcaagtgatt ttcttacctc agcctcccga gtagctggga ttacaggtgc gtgccaccac 1920

acccggctaa ttttttgtatt ttttgtagag acggggtttt gccatgttgg ccaggctggt 1980

cttgaactcc tgacatcagg tgatccacct gccttggcct cctaaagtgc tgggattaca 2040

ggcatgagcc accgtgccca accactttat ttattttta tttttatttt taaatttcag 2100

cttctatttg aaatacaggg ggcacatata taggattgtt acatgggtat attgaactca 2160

ggtagtgatc atactaccca acaggtaggt tttcaaccca ctcccctct tttcctcccc 2220

attctagtag tgtgcagtgt ctattgttct catgtttatg tctatgtgtg ctccaggttt 2280

agctcccacc tgtaagtgag aacgtgtggt atttgatttt ctgtccctgt gttaattcac 2340

ttaggattat ggcttccagc tccattcata ttgctgtaaa ggatatgatt catttttcat 2400

ggccatgcag tattccatat tgcgtataga tcacattttc tttctttttt tttttgaca 2460

cggagtcttg ctttgctgcc taggctggag tgcagtagca cgatctcggc tcactgcaag 2520

cttcacctcc ggggttcacg tcattcttct gtctcagctt cccaagtagc tgggactaca 2580

ggcgcccgcc accacgtccg gctaattttt ttgtgtgttt ttagtagaga tgggggtttc 2640

actgtgttag ccaggatggt cttgatctcc tgaccttgtg gtccacctgc ctcggtctcc 2700

caaagtgctg ggattacagg ggtgagccac tgcgcccggc ccatatatac cacatttct 2760

ttaaccaatc caccattgat gggcaactag gtagattcca tggattccac agtttgcta 2820

ttgtgtgcag tgtggcagta gacatatgaa tgaatgtgtc tttttggtat aatgatttgc 2880

attcctttgg gtatacagtc attaatagga gtgctgggtt gaacggtggc tctgtttaaa 2940

attctttgag aatttttccaa actgtttgcc atagagagca aactaattta catttccacg 3000

aacagtatat aagcattccc ttttctccac agctttgtca tcatggtttt tttttttctt 3060

tattttaaaa aagaatatgt tgttgtttc ccagggtaca tgtgcaggat gtgcaggttt 3120

gttacatagg tagtaaacgt gagccatggt ggtttgctgc acctgtcaac ccattacctg 3180

ggtatgaagc cctgcctgca ttagctcttt tccctaatgc tctcactact gccccaccct 3240

caccctgaca gggcaaacag acaacctaca gaatgggagg aaatttttgc aatctattca 3300

tctgacaaag gtcaagaata tccagaatct acaaggaact taagcaaatt tttactttt 3360
```

FIGURE 24C

```
aataatagcc actctgactg gcgtgaaatg ctatctcatt gtggttttca tttgaatttc 3420

tctgatgatc agtgacgatg agcatttttt catatttgtt ggctgcttgt acgtcttttg 3480

agaagtgtct cttcatgcct tttggccact ttaatgggat tattttttgc tttttagttt 3540

aagttcctta tagattctgg atattagact tcttattgga tgcatagttt ctgaatactc 3600

tcttccattc tgtaggttgt ctgtttactc tattgatggc ttcttttgct gtgccgaagc 3660

atcttagttt aattagaaac cacctgccaa tgtttgtttt tgttgcaatt gcttttgggg 3720

acttagtcat aaactctttg ccaaggtctg ggtcaagaag agtatttcct aggttttctt 3780

ctagaatttt gaaagtctga atgtaaacat ttgcattttt aatgcatctt gagttagttt 3840

ttgtatatgt gaaaggtcta ctctcatttt ctttccctct ttctttcttt ctttcttttc 3900

tttctttctt tctttctttc tttctttctt tctttctttc tttctttttg tccttctttc 3960

tttctttctt tctctttctt tctctctttc tttttttttt ttgatggagt attgctctgt 4020

tgcccaggct gcagtgcagc ggcacgatct cggctcactg caacctctgc ctcctgggtt 4080

caactgattc tcctgcatca gccttccaag tagctgggat tataggcgcc cgccaccacg 4140

cccgactaat ttttgtattt ttagtagaga cggggttgtg ccatgttggc caggctggtt 4200

tgaaactcct gacctcaaac gatctgcctg ccttggcctc ccaaagtgct gggattacag 4260

gtgtgagcca ctgtgcccag ccaagaatgt cattttctaa gaggtccaag aacctcaaga 4320

tattttggga ccttgagaag agaggaattc atacaggtat tacaagcaca gcctaatggc 4380

aaatctttgg catggcttgg cttcaagact ttaggctctt aaaagtcgaa tccaaaaatt 4440

tttataaaag ctccagctaa gctaccttaa aaggggcctg tatggctgat cactcttctt 4500

gctatacttt acacaaataa acaggccaaa tataatgagg ccaaaattta ttttgcaaat 4560

aaattggtcc tgctatgatt tactcttggt aagaacaggg aaaatagaga aaaatttaga 4620

ttgcatctga ccttttttc tgaatttttta tatgtgccta caatttgagc taaatcctga 4680

attattttct ggttgcaaaa actctctaaa gaagaacttg gttttcattg tcttcgtgac 4740

acatttatct ggctctttac tagaacagct ttcttgtttt tggtgttcta gcttgtgtgc 4800

cttacagttc tactcttcaa attattgtta tgtgtatctc atagttttcc ttcttttgag 4860

aaaactgaag ccatggtatt ctgaggacta gagatgactc aacagagctg gtgaatctcc 4920

tcatatgcaa tccactgggc tcgatctgct tcaaattgct gatgcactgc tgctaaagct 4980

atacatttaa aaccctcact aaaggatcag ggaccatcat ggaagaggag gaaacatgaa 5040

attgtaagag ccagattcgg ggggtagagt gtggaggtca gagcaactcc accttgaata 5100
```

FIGURE 24D

```
agaaggtaaa gcaacctatc ctgaaagcta acctgccatg gtggcttctg attaacctct 5160

gttctaggaa gactgacagt ttgggtctgt gtcattgccc aaatctcatg ttaaattgta 5220

atccccagtg ttcggaggtg ggacttggtg gtaggtgatt cggtcatggg agtagatttt 5280

cttctttgtg gtgttacagt gatagtgagt gagttctcgt gagatctggt catttaaaag 5340

tgtgtggccc ctcccctccc tctcttggtc ctcctactgc catgtaagat acctgctcct 5400

gctttgcctt ctaccataag taaaagcccc ctgaggcctc cccagaagca gatgccacca 5460

tgcttcctgt acagcctgca gaaccatcag ccaattaaac ctcttttctg tataaattac 5520

cagtcttgag tatctcttta cagcagtgtg agaacggact aatacaaggg tctccaaaat 5580

tccaagttta tgtattcttt cttgccaaat agcaggtatt taccataaat cctgtcctta 5640

ggtcaaacaa ccttgatggc atcgtacttc aattgtctta cacattcctt ctgaatgact 5700

cctcccctat ggcatataag ccctgggtct tgggggataa tggcagaggg gtccaccatc 5760

ttgtctggct gccacctgag acacggacat ggcttctgtt ggtaagtctc tattaaatgt 5820

ttctttctaa gaaactggat ttgtcagctt gtttctttgg cctctcagct tcctcagact 5880

ttggggtagg ttgcacaacc ctgcccacca cgaaacaaat gtttaatatg ataaatatgg 5940

atagatataa tccacataaa taaaagctct tggagggccc tcaataattg ttaagagtgt 6000

aaatgtgtcc aaagatggaa aatgtttgag aactactgtc ccagagattt tcctgagttc 6060

tagagtgtgg gaatatagaa cctggagctt ggcttcttca gcctagaatc aggagtatgg 6120

ggctgaagtc tgaagcttgg cttcagcagt tggggttgg cttccggagc acatatttga 6180

catgttgcga ctgtgatttg gggtttggta tttgctctga atcctaatgt ctgtccttga 6240

ggcatctaga atctgaaatc tgtggtcaga actctattat cttgagtagg acatctccag 6300

tcctggttct gccttctagg gctggagtct gtagtcagtg acccggtctg gcatttcaac 6360

ttcatataca gtgggctatc ttttggtcca tgtttcaacc aaacaaccga ataaaccatt 6420

agaacctttc cccacttccc tagctgcaat gttaaaccta ggatttctgt ttaataggtt 6480

catatgaata atttcagcct gatccaactt tacattcctt ctaccgttat tctacaccca 6540

ccttaaaaat gcattcccaa tatattccct ggattctacc tatatatggt aatcctggct 6600

ttgccagttt ctagtgcatt aacatacctg acttacattc ttttacttta aagtggaaat 6660

aagagtccct ctgcagagtt caggagttct caagatggcc cttacttctg acatcaattg 6720

agatttcaag ggagtcgcca agatcatcct caggttcagt gattgctggt agccctcata 6780
```

FIGURE 24E

```
taactcaatg aaagctgtta tgctcatggc tatggtttat tacagcaaaa gaatagagat 6840

gaaaatctag caagggaaga gttgcatggg gcaaagacaa ggagagctcc aagtgcagag 6900

attcctgttg ttttctccca gtggtgtcat ggaaagcagt atcttctcca tacaatgatg 6960

tgtgataata ttcagtgtat tgccaatcag ggaactcaac tgagccttga ttatattgga 7020

gcttggttgc acagacatgt cgaccacctt catggctgaa ctttagtact tagcccctcc 7080

agacgtctac agctgatagg ctgtaaccca agattgtcac cataaatcac attgttagac 7140

tatccagtgt ggcccaagct cccgtgtaaa cacaggcact ctaaacaggc aggatatttc 7200

aaaagcttag agatgacctc ccaggagctg aatgcaaaga cctggcctct ttgggcaagg 7260

agaatccttt accgcacact ctccttcaca gggttattgt gaggatcaaa tgtggtcatg 7320

tgtctgagac accagcacat gtctggctgt ggagagtgac ttctatgtgt gctaacattg 7380

ctgagtgcta agaaagtatt aggcatggct ttcagcactc acagatgctc atctaatcct 7440

cacaacatgg ctacagggtg ggcactacta gcctcatttg acagaggaaa ggactgtgga 7500

taagaagggg gtgaccaata ggtcagagtc attctggatg caaggggctc cagaggacca 7560

tgattagaca ttgtctgcag agaaattatg gctggatgtc tctgccccgg aaaggggat 7620

gcactttcct tgacccccta tctcagatct tgactttgag gttatctcag acttcctcta 7680

tgataccagg agcccatcat aatctctctg tgtcctctcc ccttcctcag tcttactgcc 7740

cactcttccc agctccatct ccagctggcc aggtgtagcc acagtaccta actctttgca 7800

gagaactata aatgtgtatc ctacagggga gaaaaaaaa aagaactctg aaagagctga 7860

cattttaccg acttgcaaac acataagcta acctgccagt tttgtgctgg tagaactcat 7920

gagactcctg ggtcagaggc aaaagatttt attacccaca gctaaggagg cagcatgaac 7980

tttgtgttca catttgttca ctttgccccc caattcatat gggatgatca gagcagttca 8040

ggtggatgga cacaggggtt tgtggcaaag gtgagcaacc taggcttaga aatcctcaat 8100

cttataagaa ggtactagca aacttgtcca gtctttgtat ctgacggaga tattatcttt 8160

ataattgggt tgaaagcaga cctactctgg aggaacatat tgtatttatt gtcctgaaca 8220

gtaaacaaat ctgctgtaaa atagacgtta actttattat ctaaggcagt aagcaaacct 8280

agatctgaag gcgataccat cttgcaaggc tatctgctgt acaaatatgc ttgaaaagat 8340

ggtccagaaa agaaacggt attattgcct ttgctcagaa gacacacaga aacataagag 8400

aaccatggaa aattgtctcc caacactgtt cacccagagc cttccactct tgtctgcagg 8460

acagtcttaa catcccatca ttagtgtgtc taccacatct ggcttcaccg tgcctaacca 8520
```

FIGURE 24F

```
agatttctag gtccagttcc ccaccatgtt tggcagtgcc ccactgccaa ccccagaata 8580

agggagtgct cagaattccg aggggacatg ggtggggatc agaacttctg ggcttgagtg 8640

cagaggggc ccatactcct tggttccgaa ggaggagag gctggaggtg aatgtccttg 8700

gaggggagga atgtgggttc tgaactctta aatccccaag ggaggagact ggtaaggtcc 8760

cagcttccga ggtactgacg tgggaatggc ctgagaggtc taagaatccc gtatcctcgg 8820

gaaggagggg ctgaaattgt gaggggttga gttgcagggg tttgttagct tgagactcct 8880

tggtgggtcc ctgggaagca aggactggaa ccattggctc cagggtttgg tgtgaaggta 8940

atgggatctc ctgattctca aagggtcaga ggactgagag ttgcccatgc tttgatcttt 9000

ccatctactc cttactccac ttgagggtaa tcacctactc ttctagttcc acaagagtgc 9060

gcctgcgcga gtataatctg cacatgtgcc atgtcccgag gcctgggca tcatccactc 9120

atcattcagc atctgcgcta tgcgggcgag gccggcgcca tgacgtcatg tagctgcgac 9180

tatccctgca gcgcgcctct cccgtcacgt cccaaccatg gagctgtgga cgtgcgtccc 9240

ctggtggatg tggcctgcgt ggtgccaggc cggggcctgg tgtccgataa agatcctaga 9300

accacaggaa accaggactg aaaggtgcta gagaatggcc atatgtcgct gtccatgaaa 9360

tctcaaggac ttctgggtgg agggcacagg agcctgaact tacgggtttg ccccagtcca 9420

ctgtcctccc aagtgagtct cccagatacg aggcactgtg ccagcatcag cttcatctgt 9480

accacatctt gtaacaggga ctacccagga ccctgatgaa caccatggtg tgtgcaggaa 9540

gaggggtga aggcatggac tcctgtgtgg tcagagccca gaggggccca tgacgggtgg 9600

ggaggaggct gtggactggc tcgagaagtg ggatgtggtt gtgtttgatt tcctttggcc 9660

agataaagtg ctggatatag cattgaaaac ggagtatgaa gaccagttag aatggagggt 9720

caggttggag ttgagttaca gatggggtaa aattctgctt ccgatgagtt tggggattgg 9780

caatctaaag gtggtttggg atggcatggc tttgggatgg aaataggttt gtttttatgt 9840

tggctgggaa gggtgtgggg attgaattgg ggatgaagta ggcttagttt tggagataga 9900

atacatggag ctggctattg catgcgagga tgtgcattag ttcggtttga tctttaaata 9960

aaggaggcta ttagggttgt cttgaattag attaagttgt gttgggttga tgggttgggc 10020

ttgtgggtga tgtggttgga ttgggctgtg ttaaattggt ttgggtcagg ttttggttga 10080

ggttatcatg gggatgagga tatgcttggg acatggattc aggtggttct cattcaagct 10140

gaggcaaatt tcctttcaga cggtcattcc agggaacgag tggttgtgtg ggggaaatca 10200
```

FIGURE 24G

```
ggccactggc tgtgaatatc cctctatcct ggtcttgaat tgtgattatc tatgtccatt 10260

ctgtctcctt cactgtactt ggaattgatc tggtcattca gctggaaatg ggggaagatt 10320

ttgtcaaatt cttgagacac agctgggtct ggatcagcgt aagccttcct tctggtttta 10380

ttgaacagat gaaatcacat ttttttttttc aaaatcacag aaatcttata gagttaacag 10440

tggactctta taataagagt taacaccagg actcttattc ttgattcttt tctgagacac 10500

caaaatgaga tttctcaatg ccaccctaat totttttttt tttttttttt ttttgagac 10560

acagtctggg tcttttgctc tgtcactcag gctggagcgc agtggtgtga tcatagctca 10620

ctgaacccctt gacctcctgg acttaaggga tcctcctgct tcagcctcct gagtagatgg 10680

ggctacaggt gcttgccacc acacctggct aattaaattt tttttttttt tttgtagaga 10740

aagggtctca ctttgttgcc ctggctgatc ttgaacttct gacttcaagt gattcttcag 10800

ccttggactc ccaaagcact gggattgctg gcatgagcca ctcaccgtgc ctggcttgca 10860

gcttaatctt ggagtgtata aacctggctc ctgatagcta gacatttcag tgagaaggag 10920

gcattggatt ttgcatgagg acaattctga cctaggaggg caggtcaaca ggaatccccg 10980

ctgtacctgt acgttgtaca ggcatggaga atgaggagtg aggaggccgt accggaaccc 11040

catattgttt agtggacatt ggattttgaa ataatatggga acttggtctg ggagagtcat 11100

atttctggat tggacaatat gtggtatcac aaggttttat gatgaggag aaatgtatgt 11160

ggggaaccat tttctgagtg tggaagtgca agaatcagag agtagctgaa tgccaacgct 11220

tctatttcag gaacatggta agttggaggt ccagctctcg ggctcagacg ggtataggga 11280

ccaggaagtc tcacaatccg atcattctga tatttcaggg catattaggt ttggggtgca 11340

aaggaagtac ttgggactta ggcacatgag actttgtatt gaaaatcaat gattggggct 11400

ggccgtggtg ctcacgcctg taatctcatc actttgggag accgaagtgg gaggatggct 11460

tgatctcaag agttggacac cagcctaggc aacatggcca gaccctctct ctacaaaaaa 11520

attaaaaatt agctggatgt ggtggtgcat gcttgtggtc tcagctatcc tggaggctga 11580

gacaggagaa tcggttgagt ctgggagttc aaggctacag ggagctgcga tcacgccgct 11640

gcactccagc ctgggaaaca gagtgagact gtctcagaat ttttttaaaa aagaatcagt 11700

gatcatccca acccctgttg ctgttcatcc tgagcctgcc ttctctggct ttgttcccta 11760

gatcacatct ccatgatcca taggccctgc ccaatctgac ctcacaccgt gggaatgcct 11820

ccagactgat ctagtatgtg tggaacagca agtgctggct ctccctcccc ttccacagct 11880

ctgggtgtgg gaggggggttg tccagcctcc agcagcatgg ggagggcctt ggtcagcatc 11940
```

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FIGURE 24H

```
taggtgccaa cagggcaagg gcggggtcct ggagaatgaa ggctttatag ggctcctcag 12000

ggaggccccc cagccccaaa ctgcaccacc tggccgtgga caccggt              12047
```

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FIGURE 25

```
cgagcggccc ctcagcttcg gcgcccagcc ccgcaaggct cccggtgacc actagagggc 60

gggaggagct cctggccagt ggtggagagt ggcaaggaag gaccctaggg ttcatcggag 120

cccaggttta ctcccttaag tggaaatttc ttcccccact cctccttggc tttctccaag 180

gagggaaccc aggctgctgg aaagtccggc tggggcgggg actgtgggtt caggggagaa 240

cggggtgtgg aacgggacag ggagcggtta gaagggtggg gctattccgg gaagtggtgg 300

ggggagggag cccaaaacta gcacctagtc cactcattat ccagccctct tatttctcgg 360

ccgctctgct tcagtggacc cggggagggc ggggaagtgg agtgggagac ctaggggtgg 420

gcttcccgac cttgctgtac aggacctcga cctagctggc tttgttcccc atccccacgt 480

tagttgttgc cctgaggcta aaactagagc ccaggggccc caagttccag actgcccctc 540

ccccctcccc cggagccagg gagtggttgg tgaaaggggg aggccagctg gagaacaaac 600

gggtagtcag ggggttgagc gattagagcc cttgtaccct acccaggaat ggttggggag 660

gaggaggaag aggtaggagg taggggaggg ggcggggttt tgtcacctgt cacctgctcg 720

ctgtgcctag ggcgggcggg cggggagtgg ggggaccggt ataaagcggt aggcgcctgt 780

gcccgctcca cctctcaagc agccagcgcc tgcctgaatc tgttctgccc cctccccacc 840

catttcacca ccaccatg                                                858
```

FIGURE 26

```
aagcttccac aagtgcattt agcctctcca gtattgctga tgaatccaca gttcaggttc 60

aatggcgttc aaaacttgat caaaaatgac cagactttat attcttacac caacatctat 120

ctgattggag gaatggataa tagtcatcat gtttaaacat ctaccattcc agttaagaaa 180

atatgatagc atcttgttct tagtcttttt cttaataggg acataaagcc cacaaataaa 240

aatatgcctg aagaatggga caggcattgg gcattgtcca tgcctagtaa agtactccaa 300

gaacctattt gtatactaga tgacacaatg tcaatgtctg tgtacaactg ccaactggga 360

tgcaagacac tgcccatgcc aatcatcctg aaaagcagct ataaaaagca ggaagctact 420
                                                                    454
ctgcaccttg tcagtgaggt ccagatacct acag
```

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FIGURE 27

```
GCATTGCTGT GAACTCTGTA CTTAGGACTA AACTTTGAGC AATAACACAC ATAGATTGAG
60

GATTGTTTGC TGTTAGCATA CAAACTCTGG TTCAAAGCTC CTCTTTATTG CTTGTCTTGG
120

AAAATTTGCT GTTCTTCATG GTTTCTCTTT TCACTGCTAT CTATTTTTCT CAACCACTCA
180

CATGGCTACA ATAACTGTCT GCAAGCTTAT GATTCCCAAA TATCTATCTC TAGCCTCAAT
240

CTTGTTCCAG AAGATAAAAA GTAGTATTCA AATGCACATC AACGTCTCCA CTTGGAGGGC
300

TTAAAGACGT TTCAACATAC AAACCGGGGA GTTTTGCCTG GAATGTTTCC TAAAATGTGT
360

CCTGTAGCAC ATAGGGTCCT CTTGTTCCTT AAAATCTAAT TACTTTTAGC CCAGTGCTCA
420

TCCCACCTAT GGGGAGATGA GAGTGAAAAG GGAGCCTGAT TAATAATTAC ACTAAGTCAA
480

TAGGCATAGA GCCAGGACTG TTTGGGTAAA CTGGTCACTT TATCTTAAAC TAAATATATC
540

CAAAACTGAA CATGTACTTA GTTACTAAGT CTTTGACTTT ATCTCATTCA TACCACTCAG
600

CTTTATCCAG GCCACTTATG AGCTCTGTGT CCTTGAACAT AAAATACAAA TAACCGCTAT
660

GCTGTTAATT ATTGGCAAAT GTCCCATTTT CAACCTAAGG AAATACCATA AAGTAACAGA
720

TATACCAACA AAAGGTTACT AGTTAACAGG CATTGCCTGA AAAGAGTATA AAAGAATTTC
780

AGCATGATTT TCCATATTGT GCTTCCACCA CTGCCAATAA CA
822
```

FIGURE 28A

GAATTCTTAG AAATATGGGG GTAGGGGTGG TGGTGGTAAT TCTGTTTTCA CCCCATAGGT
60

GAGATAAGCA TTGGGTTAAA TGTGCTTTCA CACACACATC ACATTTCATA AGAATTAAGG
120

AACAGACTAT GGGCTGGAGG ACTTTGAGGA TGTCTGTCTC ATAACACTTG GGTTGTATCT
180

GTTCTATGGG GCTTGTTTTA AGCTTGGCAA CTTGCAACAG GGTTCACTGA CTTTCTCCCC
240

AAGCCCAAGG TACTGTCCTC TTTTCATATC TGTTTTGGGG CCTCTGGGGC TTGAATATCT
300

GAGAAAATAT AAACATTTCA ATAATGTTCT GTGGTGAGAT GAGTATGAGA GATGTGTCAT
360

TCATTTGTAT CAATGAATGA ATGAGGACAA TTAGTGTATA AATCCTTAGT ACAACAATCT
420

GAGGGTAGGG GTGGTACTAT TCAATTTCTA TTTATAAAGA TACTTATTTC TATTTATTTA
480

TGCTTGTGAC AAATGTTTTG TTCGGGACCA CAGGAATCAC AAAGATGAGT CTTTGAATTT
540

AAGAAGTTAA TGGTCCAGGA ATAATTACAT AGCTTACAAA TGACTATGAT ATACCATCAA
600

ACAAGAGGTT CCATGAGAAA ATAATCTGAA AGGTTTAATA AGTTGTCAAA GGTGAGAGGG
660

CTCTTCTCTA GCTAGAGACT AATCAGAAAT ACATTCAGGG ATAATTATTT GAATAGACCT
720

TAAGGGTTGG GTACATTTTG TTCAAGCATT GATGGAGAAG GAGAGTGAAT ATTTGAAAAC
780

ATTTTCAACT AACCAACCAC CCAATCCAAC AAACAAAAAA TGAAAGAAT CTCAGAAACA
840

GTGAGATAAG AGAAGGAATT TTCTCACAAC CCACACGTAT AGCTCAACTG CTCTGAAGAA
900

GTATATATCT AATATTTAAC ACTAACATCA TGCTAATAAT GATAATAATT ACTGTCATTT
960

TTTAATGTCT ATAAGTACCA GGCATTTAGA AGATATTATT CCATTTATAT ATCAAAATAA
1020

FIGURE 28B

ACTTGAGGGG ATAGATCATT TTCATGATAT ATGAGAAAAA TTAAAAACAG ATTGAATTAT
1080

TTGCCTGTCA TACAGCTAAT AATTGACCAT AAGACAATTA GATTTAAATT AGTTTTGAAT
1140

CTTTCTAATA CCAAAGTTCA GTTTACTGTT CCATGTTGCT TCTGAGTGGC TTCACAGACT
1200

TATGAAAAAG TAAACGGAAT CAGAATTACA TCAATGCAAA AGCATTGCTG TGAACTCTGT
1260

ACTTAGGACT AAACTTTGAG CAATAACACA CATAGATTCA GGATTGTTTG CTGTTAGCAT
1320

ACAAACTCTG GTTCAAAGCT CCTCTTTATT GCTTGTCTTG GAAAATTTGC TGTTCTTCAT
1380

GGTTTCTCTT TTCACTGCTA TCTATTTTTC TCAACCACTC ACATGGCTAC AATAACTGTC
1440

TGCAAGCTTA TGATTCCCAA ATATCTATCT CTAGCCTCAA TCTTGTTCCA GAAGATAAAA
1500

AGTAGTATTC AAATGCACAT CAACGTCTCC ACTTGGAGGG CTTAAAGACG TTTCAACATA
1560

CAAACCGGGG AGTTTTGCCT GGAATGTTTC CTAAAATGTG TCCTGTAGCA CATAGGGTCC
1620

TCTTGTTCCT TAAAATCTAA TTACTTTTAG CCCAGTGCTC ATCCCACCTA TGGGGAGATG
1680

AGAGTGAAAA GGGAGCCTGA TTAATAATTA CACTAAGTCA ATAGGCATAG AGCCAGGACT
1740

GTTTGGGTAA ACTGGTCACT TTATCTTAAA CTAAATATAT CCAAAACTGA ACATGTACTT
1800

AGTTACTAAG TCTTTGACTT TATCTCATTC ATACCACTCA GCTTTATCCA GGCCACTTAT
1860

TTGACAGTAT TATTGCGAAA ACTTCCTAAC TGGTCTCCTT ATCATAGTCT TATCCCCTTT
1920

TGAAACAAAA GAGACAGTTT CAAAATACAA ATATGATTTT TATTAGCTCC CTTTTGTTGT
1980

CTATAATAGT CCCAGAAGGA GTTATAAACT CCATTTAAAA ·AGTCTTTGAG ATGTGGCCCT
2040

TGCCAACTTT GCCAGGAATT CCCAATATCT AGTATTTTCT ACTATTAAAC TTTGTGCCTC
2100

TTCAAAACTG CATTTTCTCT CATTCCCTAA GTGTGCATTG TTTTCCCTTA CCGGTTGGTT
2160

FIGURE 28C

```
TTTCCACCAC CTTTTACATT TTCCTGGAAC ACTATACCCT CCCTCTTCAT TTGGCCCACC
2220

TCTAATTTTC TTTCAGATCT CCATGAAGAT GTTACTTCCT CCAGGAAGCC TTATCTGACC
2280

CCTCCAAAGA TGTCATGAGT TCCTCTTTTC ATTCTACTAA TCACAGCATC CATCACACCA
2340

TGTTGTGATT ACTGATACTA TTGTCTGTTT CTCTGATTAG GCAGTAAGCT CAACAAGAGC
2400

TACATGGTGC CTGTCTCTTG TTGCTGATTA TTCCCATCCA AAAACAGTGC CTGGAATGCA
2460

GACTTAACAT TTTATTGAAT GAATAAATAA AACCCCATCT ATCGAGTGCT ACTTTGTGCA
2520

AGACCCGGTT CTGAGGCATT TATATTTATT GATTTATTA ATTCTCATTT AACCATGAAG
2580

GAGGTACTAT CACTATCCTT ATTTTATAGT TGATAAAGAT AAAGCCCAGA GAAATGAATT
2640

AACTCACCCA AAGTCATGTA GCTAAGTGAC AGGGCAAAAA TTCAAACCAG TTCCCCAACT
2700

TTACGTGATT AATACTGTGC TATACTGCCT CTCTGATCAT ATGGCATGGA ATGCAGACAT
2760

CTGCTCCGTA AGGCAGAATA TGGAAGGAGA TTGGAGGATG ACACAAAACC AGCATAATAT
2820

CAGAGGAAAA GTCCAAACAG GACCTGAACT GATAGAAAAG TTGTTACTCC TGGTGTAGTC
2880

GCATCGACAT CTTGATGAAC TGGTGGCTGA CACAACATAC ATTGGCTTGA TGTGTACATA
2940

TTATTTGTAG TTGTGTGTGT ATTTTTATAT ATATATTTGT AATATTGAAA TAGTCATAAT
3000

TTACTAAAGG CCTACCATTT GCCAGGCATT TTTACATTTG TCCCCTCTAA TCTTTTGATG
3060

AGATGATCAG ATTGGATTAC TTGGCCTTGA AGATGATATA TCTACATCTA TATCTATATC
3120

TATATCTATA TCTATATCTA TATCTATATC TATATCTATA TATGTATATC AGAAAAGCTG
3180

AAATATGTTT TGTAAAGTTA TAAAGATTTC AGACTTTATA GAATCTGGGA TTTGCCAAAT
3240

GTAACCCCTT TCTCTACATT AAACCCATGT TGGAACAAAT ACATTATTA TTCATTCATC
3300
```

FIGURE 28D

```
AAATGTTGCT GAGTCCTGGC TATGAACCAG ACACTGTGAA AGCCTTTGGG ATATTTTGCC
3360

CATGCTTGGG CAAGCTTATA TAGTTTGCTT CATAAAACTC TATTTCAGTT CTTCATAACT
3420

AATACTTCAT GACTATTGCT TTTCAGGTAT TCCTTCATAA CAAATACTTT GGCTTTCATA
3480

TATTTGAGTA AAGTCCCCCT TGAGGAAGAG TAGAAGAACT GCACTTTGTA AATACTATCC
3540

TGGAATCCAA ACGGATAGAC AAGGATGGTG CTACCTCTTT CTGGAGAGTA CGTGAGCAAG
3600

GCCTGTTTTG TTAACATGTT CCTTAGGAGA CAAAACTTAG GAGAGACACG CATAGCAGAA
3660

AATGGACAAA AACTAACAAA TGAATGGGAA TTGTACTTGA TTAGCATTGA AGACCTTGTT
3720

TATACTATGA TAAATGTTTG TATTTGCTGG AAGTGCTACT GACGGTAAAC CCTTTTTGTT
3780

TAAATGTGTG CCCTAGTAGC TTGCAGTATG ATCTATTTTT TAAGTACTGT ACTTAGCTTA
3840

TTTAAAAATT TTATGTTTAA AATTGCATAG TGCTCTTTCA TTGAAGAAGT TTTGAGAGAG
3900

AGATAGAATT AAATTCACTT ATCTTACCAT CTAGAGAAAC CCAATGTTAA AACTTTGTTG
3960

TCCATTATTT CTGTCTTTTA TTCAACATTT TTTTTAGAGG GTGGGAGGAA TACAGAGGAG
4020

GTACAATGAT ACACAAATGA GAGCACTCTC CATGTATTGT TTTGTCCTGT TTTTCAGTTA
4080

ACAATATATT ATGAGCATAT TTCCATTTCA TTAAATATTC TTCCACAAAG TTATTTTGAT
4140

GGCTGTATAT CACCCTACTT TATGAATGTA CCATATTAAT TTATTTCCTG GTGTGGGTTA
4200

TTTGATTTTA TAATCTTACC TTTAGAATAA TGAAACACCT GTGAAGCTTT AGAAAATACT
4260

GGTGCCTGGG TCTCAACTCC ACAGATTCTG ATTTAACTGG TCTGGGTTAC AGACTAGGCA
4320

TTGGGAATTC AAAAAGTTCC CCCAGTGATT CTAATGTGTA GCCAAGATCG GGAACCCTTG
4380

TAGACAGGGA TGATAGGAGG TGAGCCACTC TTAGCATCCA TCATTTAGTA TTAACATCAT
4440
```

FIGURE 28E

CATCTTGAGT TGCTAAGTGA ATGATGCACC TGACCCACTT TATAAAGACA CATGTGCAAA
4500

TAAAATTATT ATAGGACTTG GTTTATTAGG GCTTGTGCTC TAAGTTTTCT ATGTTAAGCC
4560

ATACATCGCA TACTAAATAC TTTAAAATGT ACCTTATTGA CATACATATT AAGTGAAAAG
4620

TGTTTCTGAG CTAAACAATG ACAGCATAAT TATCAAGCAA TGATAATTTG AAATGAATTT
4680

ATTATTCTGC AACTTAGGGA CAAGTCATCT CTCTCAATTT TTTGTACTTT GAGAGTATTT
4740

GTTATATTTG CAAGATGAAG AGTCTGAATT GGTCAGACAA TGTCTTGTGT GCCTGGCATA
4800

TGATAGGCAT TTAATAGTTT TAAAGAATTA ATGTATTTAG ATGAATTGCA TACCAAATCT
4860

GCTGTCTTTT CTTTATGGCT TCATTAACTT AATTTGAGAG AAATTAATTA TTCTGCAACT
4920

TAGGGACAAG TCATGTCTTT GAATATTCTG TAGTTTGAGG AGAATATTTG TTATATTTGC
4980

AAAATAAAAT AAGTTTGCAA GTTTTTTTTT TCTGCCCCAA AGAGCTCTGT GTCCTTGAAC
5040

ATAAAATACA AATAACCGCT ATGCTGTTAA TTATTGGCAA ATGTCCCATT TTCAACCTAA
5100

GGAAATACCA TAAAGTAACA GATATACCAA CAAAAGGTTA CTAGTTAACA GGCATTGCCT
5160

GAAAAGAGTA TAAAAGAATT TCAGCATGAT TTTCCATATT GTGCTTCCAC CACTGCCAAT
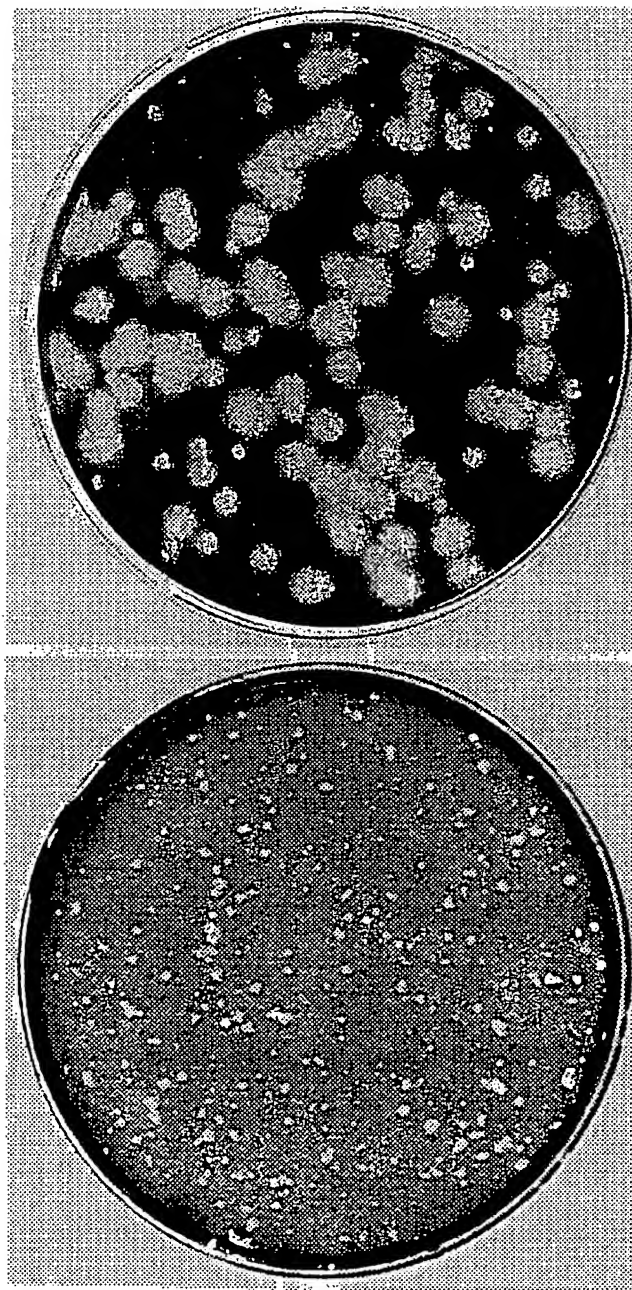5220

AACA
5224
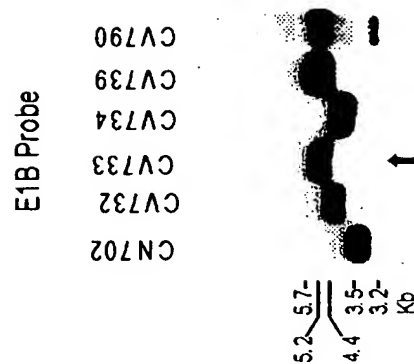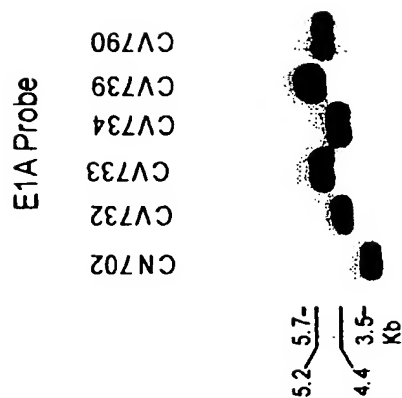
Figure 29B

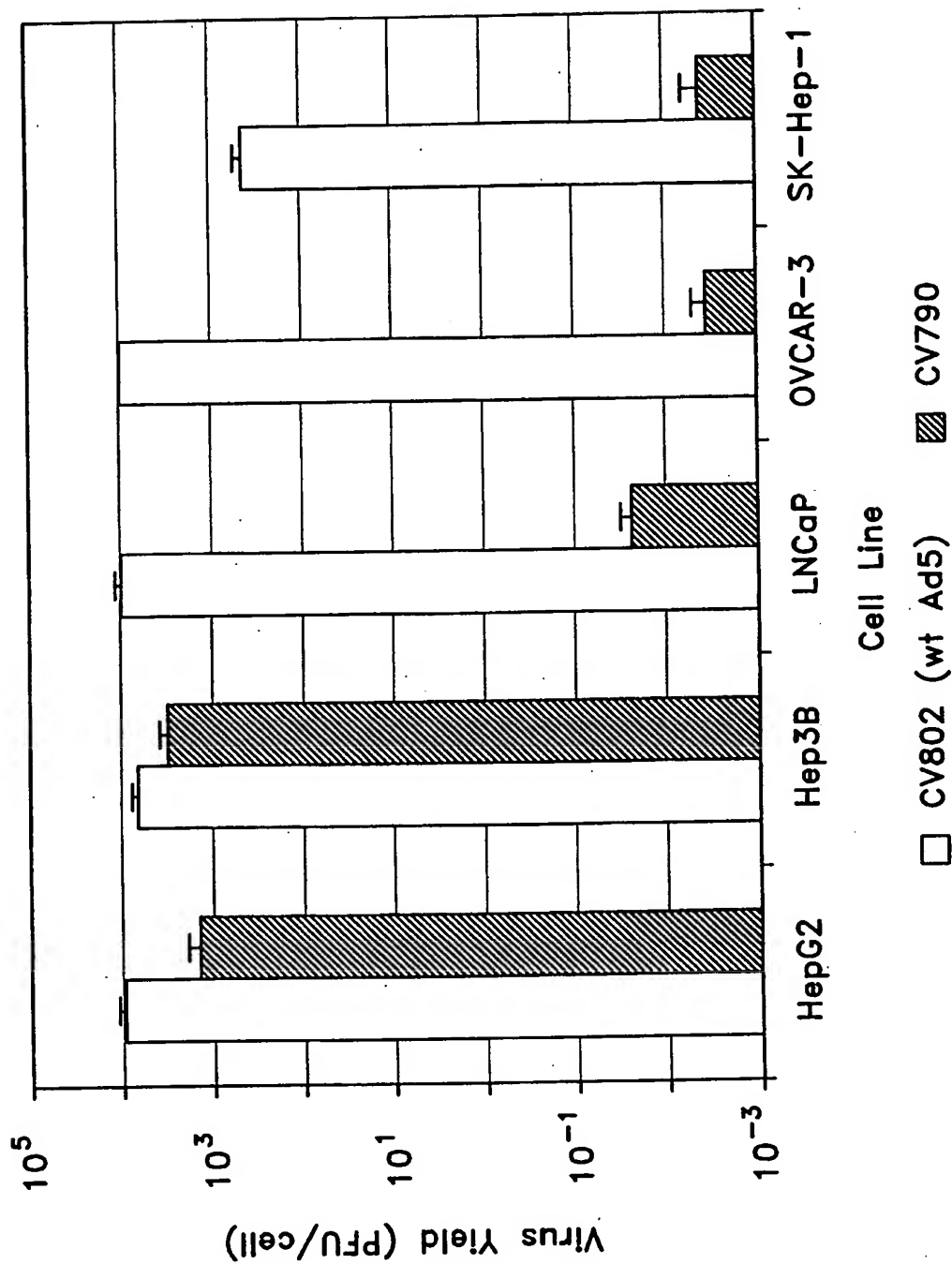Figure 29A
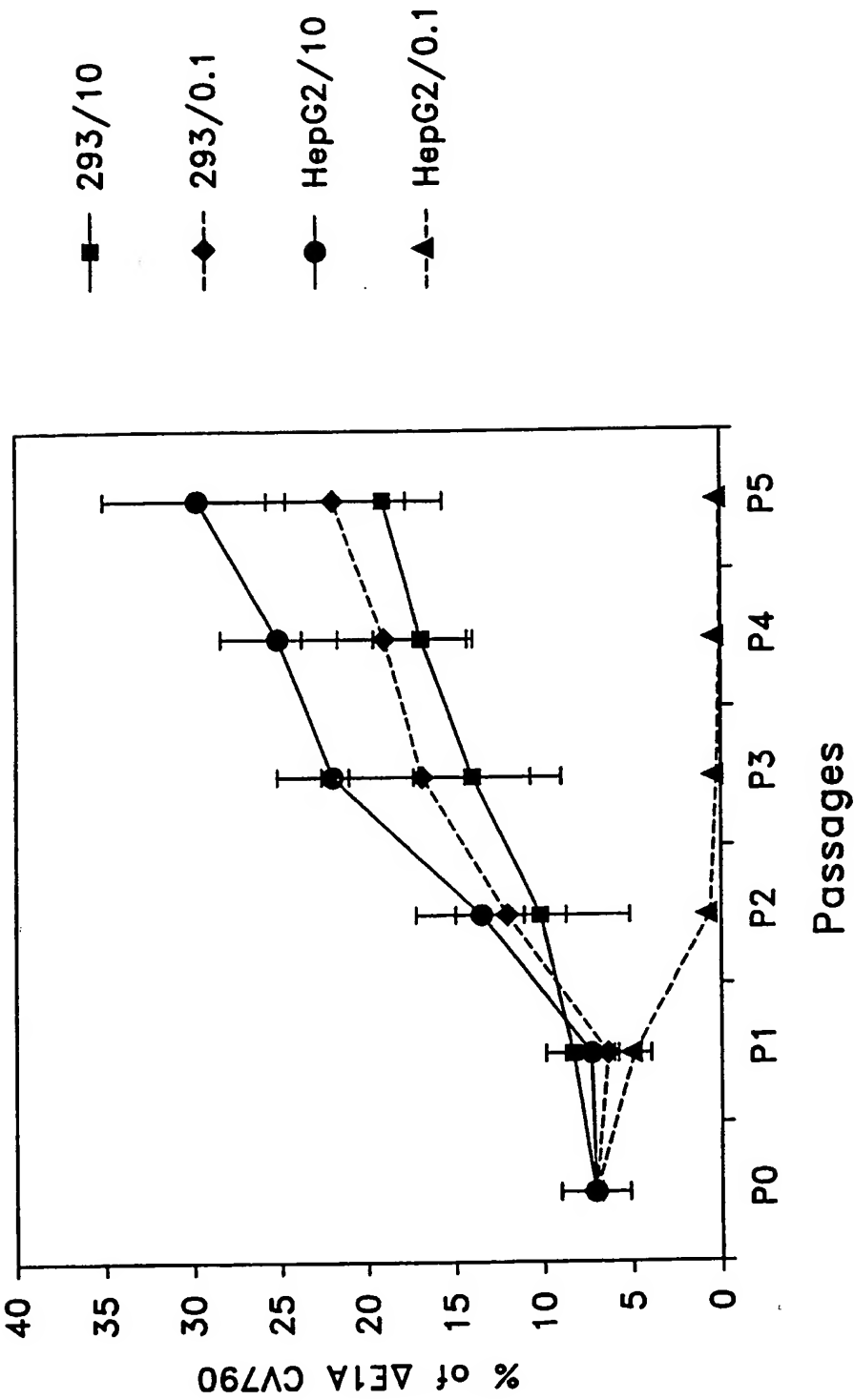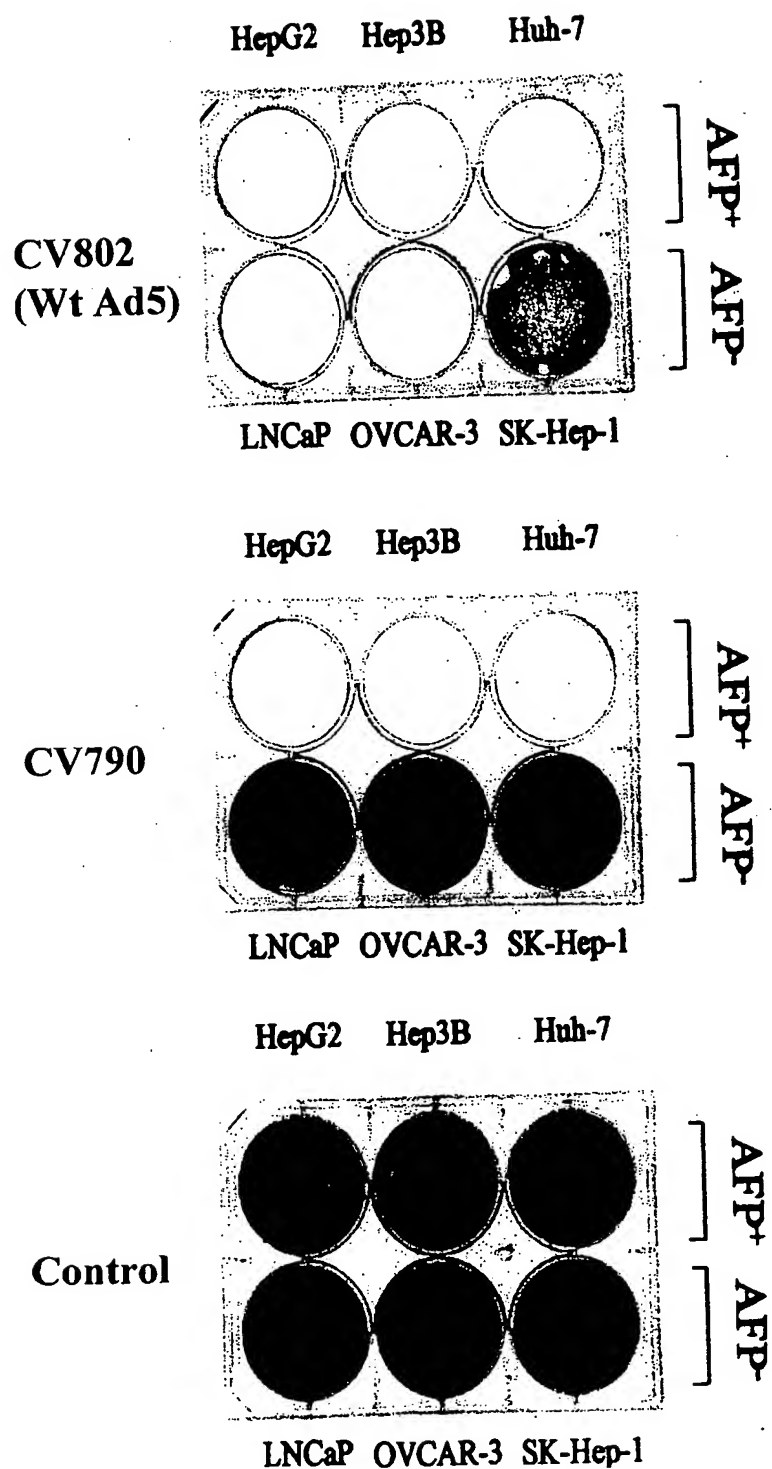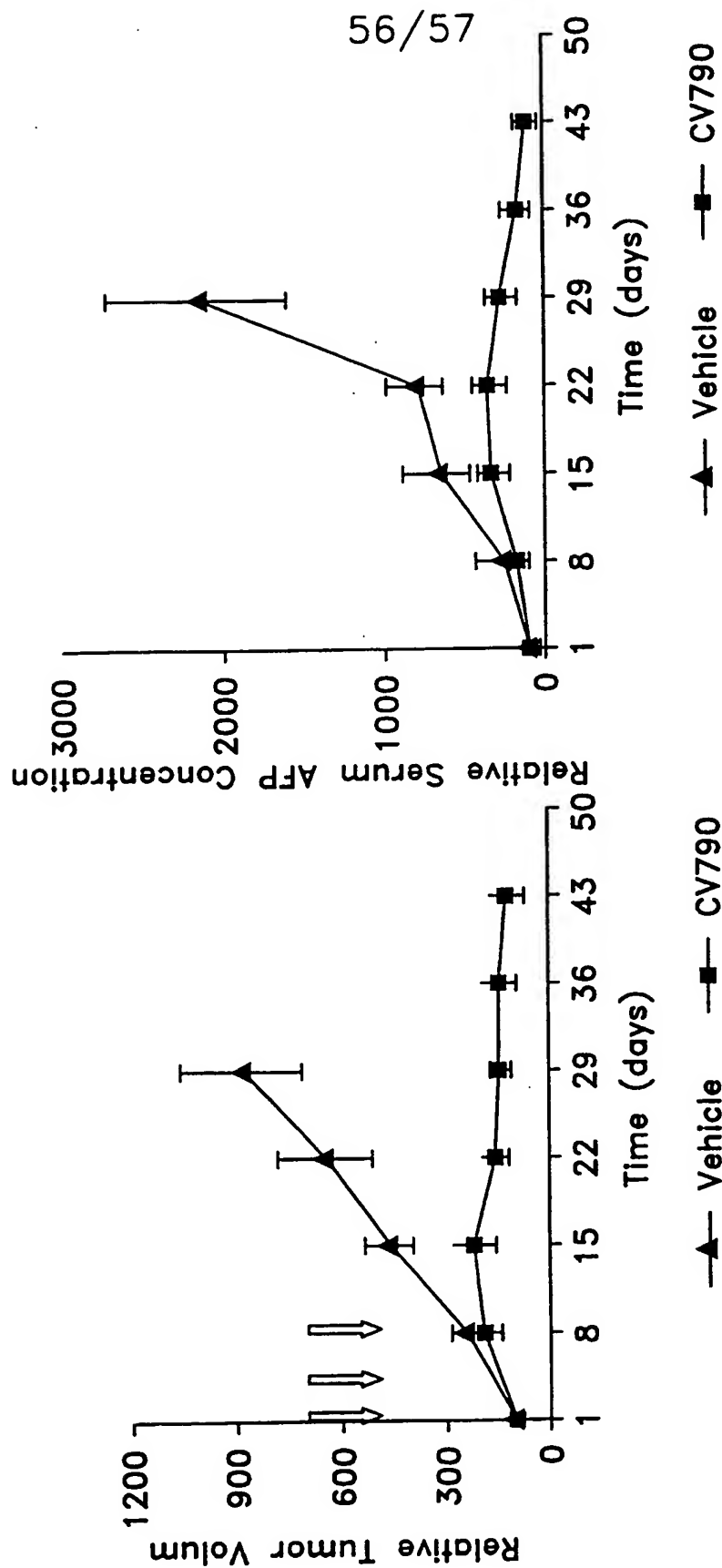
FIGURE 30

293 cells

CV790

CV733

Figure 31

FIGURE 32

FIGURE 33

FIGURE 34B

FIGURE 34A

Figure 35